

**UNDERSTANDING THE RELATIONSHIP BETWEEN
ONCOLYTIC AD5 DELETED E1b 55KDA LYTIC
INFECTION AND P53 IN MAMMALIAN CELLS**

by

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ABSTRACT

Adenoviruses are the most commonly used in the development of oncolytic therapy. Oncolytic adenoviruses are genetically modified to selectively replicate in and kill tumor cells. The p53 molecule is a tumor suppressor protein that responds to viral infection through the activation of apoptosis, which is inhibited by adenovirus E1B55kDa protein leading to progressive viral lytic cycle. The non-specificity of replication has limited the use of wild type adenovirus in cancer therapy. This issue was resolved by using an E1b deleted Ad that can only replicate in cells with a deficiency in the p53 protein, a common feature of most cancer cells.

Although demonstrating a moderate success rate, E1b55kDa deleted Ad has not been approved as a standard therapy for all cancer types. Several studies have revealed that E1b deleted Ad replication was independent of p53 status in the cell, as the virus replicated better in some p53 deficient cancers more than others. However, this mechanism has not been investigated deeply. Therefore, the objective of this study is to understand the relationship between p53 status, levels and functional activity, and oncolytic Ad5dlE1b55kDa replication efficiency.

Firstly, five transient p53 expression vectors that contain different regulatory elements were engineered and then evaluated in H1299, HEK293 and HeLa cell lines. Data indicated that vector that contains the MARs and HPRE regulatory elements achieved the highest stability of p53 expression. Secondly, we used these vectors to examine the effect of various p53 expression levels on the replication efficiency of

oncolytic Ad5dlE1b55kDa. We found that the level of p53 in the cell had an insignificant effect on the oncolytic viruses' replication. However, the functional activity of p53 had a significant effect on its replication, as Ad5dlE1b55kDa was shown to have selective activity in H1299 cells (p53-null). In contrast, a decrease in viral replication was found in HeLa cells (p53-positive).

Finally, the effect of p53's functional activity on the replication efficiency of oncolytic Ad5dlE1b55kDa was examined. Viral growth was evaluated in H1299 cells expressing number of p53 mutants. P53-R175H mutant successfully rescued viral growth by allowing the virus to exert its mechanism of selectivity. The mechanism entailed deregulating the expression of specific genes, cell cycle and apoptosis, in the p53 pathway to promote its production leading to efficient oncolytic effect. These results confirmed that oncolytic Ad5dlE1b55kDa sensitivity is mutation-type specific. Therefore, before it is applied clinically as cancer therapy for p53 deficient tumors, the type of p53 mutation must be determined for efficient antitumor effect.

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List of Abbreviations

ATM	Ataxia telangiectasia mutated
BGH polyA	Bovine growth hormone polyA signal
CAR	Coxsackie adenovirus receptor
cDNA	Complementary DNA
CPE	Cytopathic effect
CRAD	Conditionally replicative adenovirus
CTLs	Cytotoxic T lymphocytes
DBD	DNA binding domain
DBP	DNA binding protein
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EDTA-Trypsin	Ethylenediaminetetraacetic acid-Trypsin
FBS	Fetal bovine serum
FDA	Food and drug administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good manufacturing practice
HNSCC	Head and neck squamous cell carcinoma
HPRE	Hepatitis B virus posttranscriptional regulatory element
HPV	Human papillomavirus
HTA 2.0	Human transcriptome array 2.0
IDT	Integrated DNA Technology
IFN	Interferon
ITRs	Inverted terminal repeats
LB	Luria-Bertani broth
M	Mitotic
MAR	Matrix attachment region
mdm2	Mouse double minute 2
MEM	Menimum Essential Medium
MOI	Multiplicity of infection
mt	Mutant
MUTP53LOAD	Mutant p53 loss of activity database
NEB	New England Biolabs
ORFs	Open reading frames
pb	Base pair
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
Pol	Polymerase

PRD	Proline rich domain
PSA	Prostate-specific antigen
p53BS	P53 binding sites
p53RE	P53-responsive element
Rb	Retinoblastoma protein
RT	Room temperature
TAD	Transactivation domain
TB	Transformation buffer
Tm	Melting temperature
TP	Terminal protein
UV	Ultraviolet
wt	Wild type
4D	Oligomerization domain

Introduction and Literature Review

Cancer

Cancer is a rapid uncontrolled growth of cells in an organism. The abnormal cells progress together to develop a tumor as well as spread to other tissues. If spreading occurs and is not controlled, it can lead to metastasis, a major cause of death (reviewed in Chaffer and Weinberg, 2011). According to Statistics Canada 2014, the overall cancer incidence rate has been continually increasing in females whereas mostly stable in males per year over the past 30 years. Cancer accounts for nearly 30% of all deaths, making it the leading cause of death in Canada. Two in five Canadians are expected to develop cancer during their lifetime (Statistics Canada, 2014).

There are hundreds of types of cancer named for the organ where it started. The most common types are prostate, breast and lung cancer (American National Cancer Institute, 2014). Cancer develops through an accumulation of genetic mutations in genes that regulate cellular growth and division. This is caused by several factors, including external (chemicals and radiation) and internal (inherited and somatic mutations). Although some of the factors are alike, each type of cancer behaves differently (reviewed by Fearon and Vogelstein, 1990).

Steady progress has been made to improve cancer therapy since the war on cancer was launched in the 1970s. However, most metastatic tumors remain incurable (reviewed in Choi and Yun, 2013). The main reason for this is that cancer is born of the body's own

cells, making it difficult for treatments to differentiate between healthy and malignant tissues.

Cancer Therapy

Traditional Therapy

Before 1950, surgery and surgical treatment have been routinely performed to treat several cancers for many years such as colorectal carcinoma, as it results in 25-40% 5-year survival rate (Wagner, *et al.*, 1984; Perlmutter and Lepor, 2007). With the inception of radiotherapy and chemotherapy, surgical option is restricted to special cases with a clear resection margin (Ohlsson *et al.*, 1998). However, over time neither chemotherapy or radiotherapy has shown promising results due to lack of selectivity since both cancer and normal cells are killed (Johnstone *et al.*, 2002). Since the multiple side effects of chemotherapy and radiotherapy have limited the extension of the duration of therapy, development of novel approaches that are more selective and less toxic was needed.

A revolution in cancer treatment was reached with advancement in the field of cellular and molecular biology, which revealed that cancer is a genetic disease caused by genome instability that leads to abnormal cell progression. This discovery has created a novel framework for researchers to repair the molecular defect in tumor cells, beginning the era of targeted therapy.

Targeted Therapy

Targeted agents are mainly molecules that attack cancer cells but cause less damage to normal cells. Multiple targeting approaches have been investigated for their anti-tumor efficacy such as targeting angiogenesis and apoptosis. Improvements in understanding apoptosis, known as programmed cell death, has led to utilization of this system to eliminate cancer cells. Since the 1990s, many important discoveries of apoptosis related genes have opened new doors for development of apoptosis inducing drugs that are capable of triggering cell death (reviewed in Wong, 2011).

With the continuous discovery of novel cancer related genes, it has been observed that different tumors express different defective genes. The defects of these genes have attributed to the accumulation of mutations over time, which consequently results in destroying their function (Vogelstein and Kinzler, 1993). It was reported that mutation event actually occurs in the most documented key regulatory genes that are responsible for cancer occurrence and progression, tumor suppressor genes and oncogenes. These genes include tumor suppressor p53, p16INK4a (p53 stabilizer) and retinoblastoma protein (Rb) (Sherr, 1996). This finding suggests that if the expression of tumor suppressor genes in cancer cells is restored, cell growth might once again be modulated, restoring the anti-tumor effect, hence cancer gene therapy was proposed (Fujiwara *et al.*, 1993).

Gene Therapy

The term “Gene therapy” is used to describe a technology that involves transferring therapeutic molecules directly to the cells (Anderson, 1998). It has become one of the most rapidly developing areas in clinical research, and has the potential to treat numerous kinds of cancer. A number of alternative targeting strategies for cancer gene therapy have been developed. One approach aimed to increase drug selectivity towards cancer cells. As initial gene therapy focused on treating single gene defects, the identification in the early 1980s of critical tumor targets such as p53 and E2F from DNA tumor virus studies was essential (Lane and Crawford, 1979; Helin *et al.*, 1992). Tumor suppressor p53 was the first target for cancer gene therapy, as it revealed potent tumor suppressor activity against cancer. Since most tumor cells have inactive p53 protein, scientists have suggested replacing the non-functional protein in tumor cells with the intact version to restore the function (Finlay *et al.*, 1989; Levine *et al.*, 1994). Since then, all potential anti-tumor gene therapy-based agents became putative targets for gene therapy. However, this process requires a vector to deliver the therapeutic gene to the target tumor cells. For this purpose, intensive efforts have focused on engineering delivery vehicles to improve gene and drug delivery including viral and non-viral based vectors.

Although non-viral based vectors are a suitable system for gene therapy with respect to safety, easy development, and non-immunogenic features, they have limitations in gene size and show modest delivery efficiency (reviewed in Lundstrom and Boulikas, 2003). In addition, viral based vectors excel for their efficient gene delivery. This is

because viruses can efficiently infect a broad range of cells and have the ability to target specific cells. They also possess strong promoters that generate a high expression level of the therapeutic gene (reviewed in Giacca and Zacchigna, 2012). Therefore, it is not surprising that a wide range of viruses is under development to achieve excellent delivery.

Viruses in the field of gene therapy fall into one of two categories, either wild type viruses or recombinant viruses. Due to the safety concerns of using viruses in cancer gene therapy, two thirds of current clinical practices are using non-replicative viruses as part of their therapeutic strategies. These viruses are recombinant agents that have been engineered to be non-replicative through the deletion of genes responsible for viral replication. The non-replicative characterization allows the expression of therapeutic genes only in the cells they infect, providing high efficacy and clinical safety (Kurooka and Kaneda, 2007). A large variety of recombinant viruses have been developed for cancer gene therapy, including adenoviruses, Newcastle disease virus, retroviruses, and many other viruses (reviewed by Giacca and Zacchigna, 2012). Roth reached the first successful use of viruses in this field in 1996 when wild type p53-containing retrovirus was injected into non-small cell lung cancer (Roth *et al.*, 1996). A few years later, the first recombinant non-replicative adenovirus lacking the E1 and E3 regions was engineered (Andrews *et al.*, 2001).

Among the recombinant vectors, adenovirus represents the ideal gene transfer vector. Its ability to transfer genes without integration in the genome has assured the safety of this vector (Harui *et al.*, 1999). This characteristic, and several more such as its

capability of infecting dividing and non-dividing cells and stability of its genome have made significant advances in its path to clinical evaluation. To date, adenovirus-based vectors has accounted for a quarter (24.7%) of gene therapy clinical trials around the world (reviewed by Edelstein *et al.*, 2007). A well-known example is Gendicine, an Ad-p53 recombinant vector, which is the most popular approach in viral mediated gene therapy. In 2004, China's State Food and Drug Administration approved Gendicine as a treatment for head and neck cancer.

Although results of many studies using the strategies to treat different cancers agree with these findings, different recent *in vitro* and clinical studies have shown inconsistent results in terms of antitumor effect (Fujiwara *et al.*, 2006). Thus although non-replicating vectors are more safe, the shortcoming of limiting replication prevents viral spread to the entire tumor, particularly for advanced tumors (Heise *et al.*, 1999). Nevertheless, in the context of local disease, gene-vector-based therapy with repeated dosing would be a suitable choice when it is combined with other modalities such as chemo- and radio-therapy (Pan *et al.*, 2009).

Virotherapy

The concept of virotherapy is about 100 years old, and began when DePace observed a reduction in tumor size in cervical cancer after inoculation with attenuated rabies vaccine in 1912 (reviewed in Ring, 2002). In parallel with that observation, previous report in 1904 when Dock documented tumor regression from cancer patients, while suffering from viral infection. These historical records suggest the notion that viruses may be able to eradicate cancer, and therefore may be used as cancer therapy

agents, otherwise known as oncolytic viruses. Shortly after, Levaditi and Nicolau conducted an animal experiment on mouse tumors in the 1920s to report the first oncolytic vaccinia agent for cancer therapy. They noticed impaired tumor growth of virally infected tumor whereas no noticeable reduction in uninfected tumors. Since then, during the 1950s and 1960s, a series of studies to investigate the effect of oncolytic viruses in humans have been established. These investigations have provided the scientific community with a hint that these viruses might possess a unique mechanism of anti-tumor effect that explains their capacity of tumor regression (reviewed in Kelly and Russell, 2007).

Several animal studies were conducted by several groups to test the antitumor effect of number of viruses. Among the tested viruses, the first generation of potential oncolytic viruses, which were then chosen for human clinical trials (reviewed in Meerani and Yao, 2010). Many human clinical trials with different oncolytic viruses were carried out to treat several cancers such as cervical cancer (Smith *et al.*, 1956). Despite the observations of partial and complete tumor regression, first generation naturally oncolytic viruses have elicited infectious complications that ultimately ended the trials in the late 1960s. In addition, a severe immune response has elicited as a result of expression of wild type viral proteins, which limited their success. The latter drawbacks have caused significant decline in viral oncotherapy for decades (reviewed in Kelly and Russell, 2007).

As years passed, a better understanding of virology combined with the advent of gene therapy and biotechnology concepts in the 1990s has increased the interest in

mutated viruses as cancer treatment, giving rise to the second generation of oncolytic viruses. Today, oncolytic virotherapy is the most rapidly growing area in cancer treatment. It is considered a formidable therapy option for cancer in combination with chemo- and radio-therapy (Taneja *et al.*, 2001).

Oncolytic Viruses

The term oncolytic virus refers to viruses that have the capability to selectively replicate in and destroy cancer cells (Alemany *et al.*, 2000). To date, around 675 cancer gene therapy clinical trials, where more than half of the trials have been focused on oncolytic viruses (reviewed by Aghi and Martuza, 2005). Viruses are small parasites that have the natural ability to spread, grow and kill the infected cells. Since cancer has certain molecular defects in multiple signaling pathways, such as the p53 and the interferon (IFN) pathway, these viruses can be genetically manipulated and designed to exploit the aberrant cellular and molecular pathways in cancer cells resulting in selective replication. This means that the replication and destruction ability of oncolytic viruses is restricted to cancer cells rather than normal cells (Morley *et al.*, 2004).

The mechanism of viral oncolysis relies on the lytic replication cycle. Upon infection of one cancer cell, the genetically engineered virus begins to multiply and replicate to lyse the infected cell resulting in the release of new virions that are capable of targeting and infecting the adjacent cancer cells in a tumor. After a number of cycles, the virus spreads within the cells facilitating the destruction of all tumor cells as seen in (Figure 1) (Biederer *et al.*, 2002). Beside its efficient targeting property, the replication event of many oncolytic viruses has triggered strong immune response towards the

infected cancer cells, thus functioning as an adjuvant. Therefore, viral oncolysis is considered a two-pronged strategy, oncolytic from one side and immunotherapeutic from the other side (Benencia *et al.*, 2008).

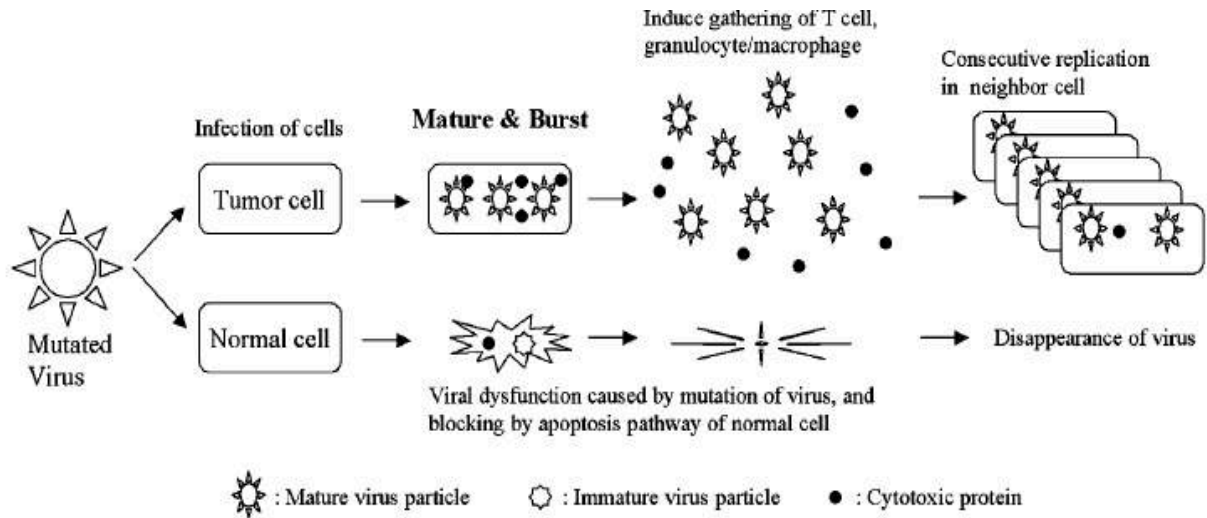


Figure 1: Mechanism of action of oncolytic viruses (Reported by Kasuya *et al.*, 2005)

Selection Criteria of Oncolytic Viruses for Cancer Therapy

Identification of the optimal virus for oncolysis is a challenging task. To consider an ideal virus species for oncolysis, it should demonstrate a number of specific characteristics. The virus needs to be safe with minimal side effects. It should be a genetically stable virus with a large gene carrying capacity and easy to engineer for both safety and manufacturing standpoints. It must be efficient by achieving complete destruction of tumor cells. It can infect dividing and non-dividing cells with available mechanisms for selective replication in cancer cells. The virus needs to be grown in high titers for clinical study production under Good Manufacturing Practice (GMP) guidelines. It should preferably not integrate in the host genome to avoid causing further mutations.

In addition, the virus must have its receptors expressed redundantly on cancer cells to facilitate viral internalization with the ability to trigger strong immune response against tumor cells, providing the two-pronged mechanism. Finally, the virus must be cost effective and easy to monitor (Vile *et al.*, 2002).

A number of viruses from many different families exhibited most of the characteristics and therefore were proposed for oncolytic development. Such viruses that were found to be safe oncolytic agents and have shown promising results in preclinical studies include adenovirus, herpes simplex virus, and vaccinia virus (Martuza *et al.* 1991; Zhang *et al.*, 2007). However, adenovirus is a better candidate for therapeutic development. Beside its match to the above criteria for an ideal oncolytic agent, adenovirus is well characterized and much is known about its biology. Also, it has a long history of usage in gene therapy as a non-replicating vector (reviewed by Yamamoto, 2004).

Oncolytic Adenovirus

Conditionally replicative adenovirus (CRAD) is a genetically modified human adenovirus that can restrict its growth to only cancer cells. Ad-ONYX-015 is the first successful adenovirus mutant that was approved for clinic use. This virus was engineered with deletion in E1B gene region to target and replicate in cells that have a defective tumor suppressor p53 pathway (Bischoff *et al.*, 1996). As a result of its efficient reduction of tumor size and inducing only mild flulike symptoms, it was approved in China in 2005 to treat head and neck squamous cell carcinoma (HNSCC) patients and nasopharyngeal cancer combined with standard therapy (Garber, 2006).

Adenoviruses

In 1953, adenovirus was first isolated from human adenoidal tissue sample in culture as was found to exist in 62% of adenoid specimens, thus named “adenovirus” (Rowe *et al.*, 1953). The different virus serotypes predominantly cause limited respiratory and gastrointestinal illnesses as well as ailments in the conjunctiva of the eye. Adenovirus serotype 5 is the most widely used in research because it belongs to the non-oncogenic sub group C. Therefore, it does not induce cancer, which makes it the most favorable for cancer research.

Classification

Adenoviruses are small DNA viruses that belong to *Adenoviridae*. The latter is subdivided into five groups according to their host as follows: Atadenovirus, Aviadenovirus, Siadenovirus, Ichtadenovirus and Mastadenovirus, in which human adenoviruses fall into. The genus Mastadenovirus is categorized into six subgroups, based on their agglutination property and their oncogenic activity, named A to F. These species are in turn divided into 51 serotypes (Benko *et al.*, 1999).

Structure

Adenovirus consists of non-enveloped icosahedral capsid with diameter of approximately 80-110 nm. The outer capsid is composed of structural proteins, hexon, penton, and fiber, as major capsid proteins along with several minor ones (V, VI, VII, VIII, IX, IIIa and IVa2) as shown in (Figure 2). Each viral particle consists of 252 capsomers, in which 240 are hexons and 12 are pentons. Hexon is the largest, as it is the

building block of 20 faces of the viral particle. Penton and fiber proteins are essential for viral internalization, in which penton binds integrins and fiber binds coxsackie adenovirus receptor (CAR) to facilitate the entrance (Russell, 2009).

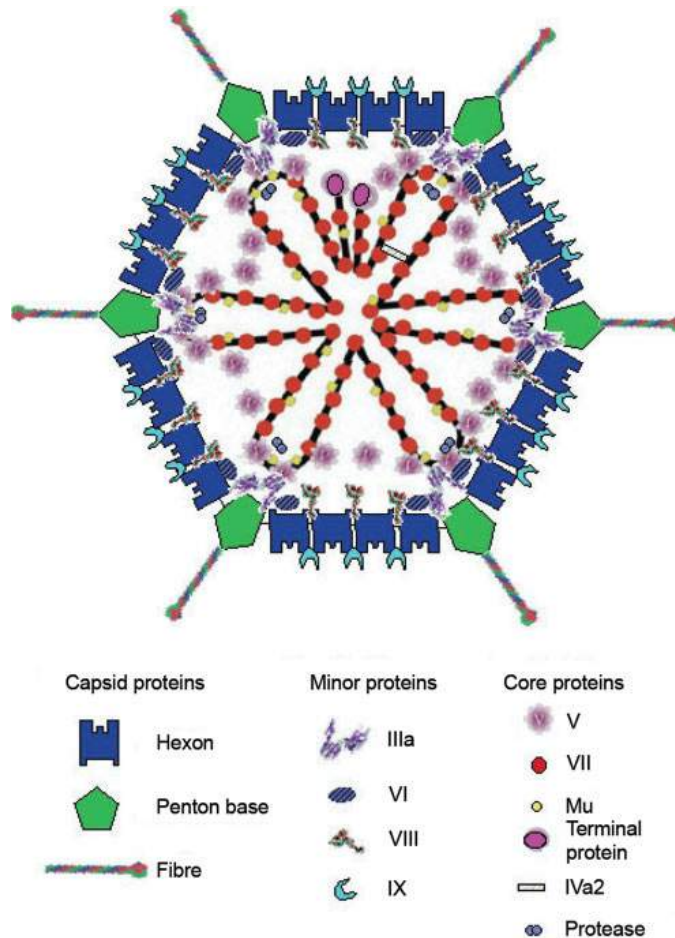


Figure 2: Structure of adenovirus. A schematic depiction of the structure based on cryo-electron microscopy and crystallography. The locations of the capsid and minor components are reasonably well defined and are not to scale. The disposition of the core proteins and the virus DNA is largely conjectural. Reported by (Russell, 2009) and the symbols for IIIa and VIII are based on the structures defined by Saban *et al.* (2006).

Genome

The genome of adenovirus is linear double-stranded DNA with a size of about 36-38kb. The genome is flanked by inverted terminal repeats (ITRs) protected by two

terminal proteins at the 5' ends (Rekosh *et al.*, 1977). The interaction of both terminal proteins is essential to maintain the genome in a quasi-circular state, therefore facilitating the replication of viral DNA (Seth, 1999). The adenovirus genome encodes proteins whose genes are divided into two groups according to their stage in transcription. The viral genes transcripts and translated proteins are shown in Figure 3.

Figure 3: Genome structure of adenoviruses (From Shenk, 1996).

Adenovirus type 5 infection cycle is divided into two distinct phases: early and late. The early phase begins with viral internalization into the cell by integrin binding through CAR-mediated endocytosis. After that, the viral genome enters the nucleus, followed by transcription and translation of early viral genes to drive infected cells into

unscheduled replication. Then, the late phase begins by a series of transcription of genes encoding structural capsid proteins, facilitating the production of large quantities of new viruses that induce cell lysis (Mathias *et al.*, 1994; Russell, 2009). As a tremendous amount of information is available on every gene and network, this review will focus only on genes that are directly related to replication and oncolysis, which is the focus of our study.

Early Genes

E1

Transcription of early genes, E1, E2, E3, and E4 begins as soon as viral DNA enters the nucleus. E1 is the first gene that is transcribed upon viral infection. It consists of two genes, E1A and E1B. E1A gene is the most essential element, as it is responsible of stimulation of viral DNA synthesis and transcription activation of other early genes (Berk *et al.*, 1979). Both genes work together to regulate apoptosis and to force the cell to enter S phase in favor of virus replication. These genes are the primary targets of modification for creating tumor selective replicative adenoviruses (Moran, 1993).

Immediate early (E1A)

E1A is the first protein produced immediately upon infection. The processing of E1A transcript yields five different products, 13S, 12S, 11S, 10S, and 9S. Their mRNAs encode for different proteins in which the major ones, 13S that encode for 289R and 12S that encode for 243R, are responsible for major functions of E1A including the transcriptional regulation of several cellular and viral proteins. Between these amino acid

residues, there are three conserved regions named, CR1, CR2, and CR3 that act to promote the multiple functions of E1A (Shenk, 2001). One critical function of E1A is regulating the transcription of other early and late genes in the infection cycle. For this reason, E1A is considered a master regulator of the adenovirus replication process.

During viral replication, adenovirus utilizes its own polymerase and other proteins required for DNA replication to synthesize its viral DNA. At the same time, E1A transforms the cell into S phase and stimulates the cell cycle progression, thus providing an optimal environment for efficient viral DNA synthesis (Whyte *et al.*, 1988; Howe *et al.*, 1990). The latter is achieved through the ability of CR1 and CR2 to bind to certain tumor suppressor proteins including retinoblastoma protein (Rb) and preventing its binding to E2F transcription factor, which controls the entry into S phase, resulting in activation of cell cycle progression genes (Hiebert, 1993). The release of E2F will lead to stabilization of tumor suppressor p53 protein that is required to implement apoptosis. This reveals another activity of E1A expression in which it can induce p53-dependent apoptosis, which must be inhibited to avoid host cell death during viral infection (White *et al.*, 1992).

The fact that CR3 is not involved in the cell transformation process does not cancel its importance. CR3 is a critical transcriptional activator of E1B, E2E, E3, and E4 and stimulates the activity of major late promoter (MLP) for efficient progression (Bruton *et al.*, 2007, 2008). Together, the E1A protein products provide a powerful mechanism for efficient viral replication through deregulating cell cycle genes.

E1B

E1B is the product of an mRNA 2,200 nucleotides in length. It contains two major proteins, as a result of translation of two distinct start codons, E1B19kDa and E1B55kDa. Both proteins contribute to cellular transformation mediated by E1A; however, they prevent the unwanted apoptosis, unlike E1A (Debbas and White, 1993). Despite the differences in their sequences and their functions, two overlapping frames encode both proteins (Esche *et al.*, 1980).

The E1B19kDa protein is found to be a homolog of Bcl-2 anti-apoptotic family in sequence as well as function. It has an anti-apoptotic effect that has shown to block apoptosis induced by multiple pro-apoptotic genes including E1A, TNF- α , Fas, p53, Bid, Bax, and pro-apoptotic mitochondrial proteins that thus inhibit caspase activation. E1B19K deleted adenovirus has shown DNA degradation as well as strong cytopathic effect in the infected cells (White *et al.*, 1984).

The larger E1B55kDa protein carries out multiple functions. The major function presents in its ability to interact with tumor suppressor protein p53 and inhibit its function on different levels. E1B55kDa binds to p53 domains and counteracts its transactivation activity such as inhibiting p53-mediated apoptosis, disrupting cell cycle checkpoint control, and losing the DNA repair system (Martin and Berk, 1999). In addition, E1B55kDa together with viral protein E4orf6 has another function, in which both form a complex with E3 ubiquitin ligase that targets p53 for proteasomal degradation. However, the absence of E4orf6 does not affect the p53 repression ability of E1B55kDa (Querido *et al.*, 1997). The mechanisms of apoptosis of E1B55kDa and E1B19kDa are shown in

(Figure 4).

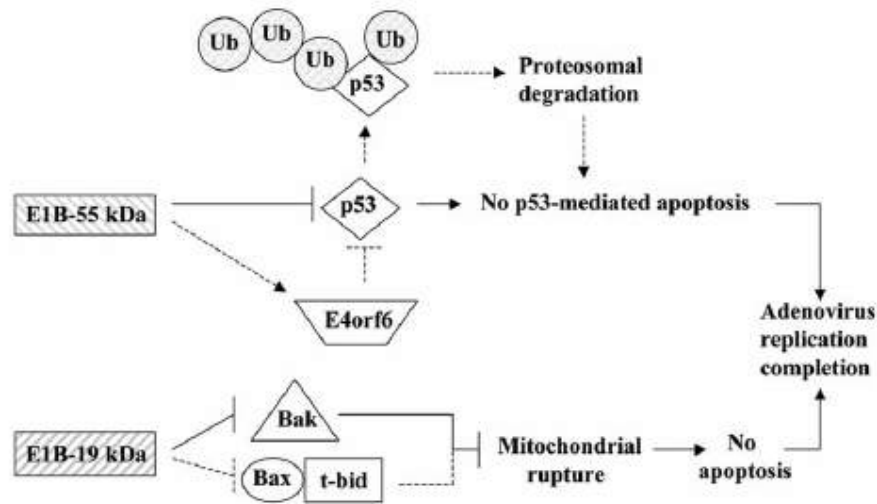


Figure 4: E1B protein anti-apoptotic mechanisms. The two region proteins, E1B55 kDa and E1B19 kDa, both result in apoptotic prevention mechanism to help ensure successful viral infection and spread. The E1B55kDa predominantly functions to sequester and inhibit p53 but can also indirectly act through E4orf6 to ubiquitinate and degrade p53 to prevent p53-activated apoptosis. The E1B19 kDa protein acts as Bcl-2 analog and inhibits Bax and/or Bak from signaling mitochondrial rupture, cytochrome c release, activation, and ultimate apoptosis (from Jounaidi *et al.*, 2007).

The complex of E1B55kDa and E4orf6 also results in the degradation of Mre11 to stop the damage response towards viral genome, thus preventing the viral genome end-joining in cooperation with ataxia telangiectasia mutated (ATM) protein kinase expression (Stracker *et al.*, 2002). Moreover, the E1B55kDa/E4orf6 complex promotes the transportation of viral mRNA from the nucleus through E4 nuclear localization and transport signalling (Dobner *et al.*, 1996). However, the same complex retains the host specific mRNA, thus blocking cellular protein synthesis (Dobbelstein *et al.*, 1997). The deletion of adenovirus E1B region restricts viral growth in the intact p53-infected cells, as allowing p53-mediated apoptosis.

E2

Expression of the E2 gene is associated with expression of E1A, as the latter activates E2 and the other early genes. E2 gene comprises about 20kb of the viral genome that is transcribed under the control of two promoters early and late to form two mRNA products from different splicing signals, E2A and E2B. Despite their differential functions, both genes encode three main proteins that are essential for initiating viral DNA replication, which are DNA binding protein (DBP), terminal protein, and viral DNA polymerase. A sufficient concentration of these proteins is required to start the replication process, which begins 5 to 8 hrs post infection. Viral DNA replication will lead to productive replication of the virus resulting in production of new virions (Shenk, 2001).

The E2A gene encodes DBP, which is about 72kDa. It has high binding affinity to single stranded DNA, which is required to achieve all functions. DBP has major functions during replication. It prevents the formation of a DNA duplex structure leaving unwinding the double stranded DNA for chain elongation (Brenkman *et al.*, 2001). It also protects against DNA degradation by nucleases, thus increasing the stability of single stranded DNA. In addition, DBP has a role in enhancing the interaction of terminal protein with DNA and the binding of polymerase to the origin of replication (Van Breukelen *et al.*, 2003).

E2B gene encodes two distinct proteins: terminal protein (TP) and viral DNA polymerase (Pol), 55kDa and 140kDa, respectively. Both proteins are involved in the initiation of replication from one parental strand of DNA. The adenovirus polymerase

uses protein primer pTP to produce double stranded DNA via a protein-priming mechanism (Parker *et al.*, 1998). Then, viral polymerase will perform the addition of nucleotides in a specific manner until the elongation step is finished. The other parental single strand forms a ‘pan-handle’ structure, annealed inverted terminal repeats (ITRs), for the second round of DNA replication using the same mechanism. These ITRs are located at the ends of each parental strand to serve as an origin of replication (Brenkman *et al.*, 2002). When the replication process is done, viral polymerase will perform the cleavage of pTP resulting in its mature form (TP) (Smart and Stillman, 1982) (Figure 5).

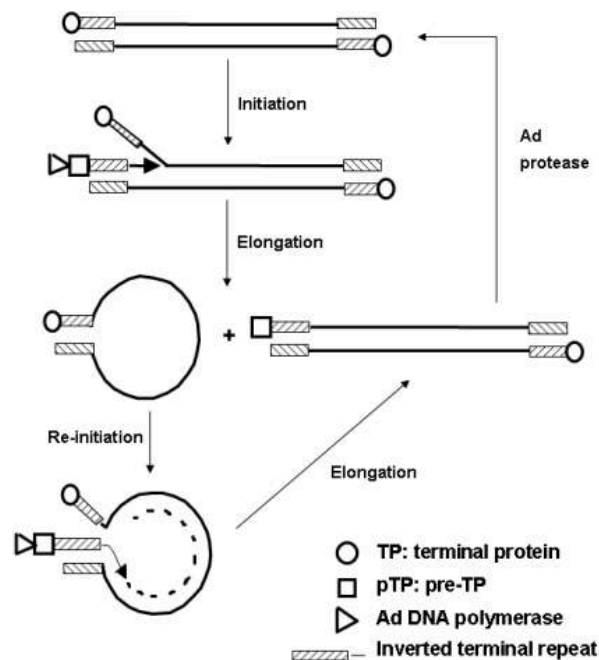


Figure 5: Adenovirus DNA replication model

E3

The region of E3 is essential to counteract the host immune response against the virus. The translation of E3 gene results in generation of several proteins with immunomodulatory functions, E3 11.6kDa, E3 14.5kDa, E3 10.4kDa, and E3-gp 19kDa.

They all participate together to reach one goal, hindering the recognition of virus proteins and eliminating the targeting of infected cells by cytotoxic T lymphocytes (CTLs). (Burgert *et al.*, 1987; Dimitrov *et al.*, 1997), thus, facilitating persistent infection of adenovirus in the infected cell. For efficient replication of oncolytic adenovirus, E3 11.6kDa death gene deletion is required. The deletion is essential for avoiding premature death during the productive adenovirus infection cycle (Tollefson *et al.*, 1996a). E3 region is dispensable for virus replication *in vitro*; therefore it is deleted in the majority of adenovirus vectors.

E4

E4 protein is a product of 18 different mRNAs, as a result of alternative splicing, that are activated by E1A-13S. E4 different transcripts, encode several open reading frames (ORFs), which are named according to their arrangement within the region as (E4orf1, E4orf2, E4orf3, E4orf4, E4orf5, E4orf6, and E4orf7) (Virtanen *et al.*, 1984). Some of the E4 proteins are functionally known whereas others lack a clear function (reviewed in Leppard, 1997).

Late Genes

The transcription of late genes begins after DNA replication under the control of the MLP. In the late phase of infection, MLP becomes fully active which results in producing a transcript of 29kb nucleotides in length. The alternative splicing of this transcript results in five distinct groups of genes. These genes are categorized based on different poly-A sites, into L1 to L5 (Nevins and Darnell, 1978). Late genes encode viral

structural proteins (IV, VI, VII, VIII, Mu) that are essential for virion assembly (Smith *et al.*, 2010).

Mechanism of Antitumor Efficacy

Adenovirus mediates oncolytic activity and tumor killing by an array of different mechanisms (reviewed by Mullen and Tanabe, 2002). The first mechanism is the result of viral replicative cycles that cause direct lysis of infected cells. The new virions then infect neighboring cancer cells resulting in subsequent destruction using the same mechanism. These cycles can be constantly repeated providing a continuous amplification of the initial dose until they are stopped by either of immune response or a rareness of susceptible cells.

The second mechanism involves direct toxicity of the infected cells through the expression of toxic viral proteins. In this regard, adenovirus can generate certain proteins during replication, which are E3 11.6kD and E4orf4 protein. Both proteins are toxic and able to damage the cell (Shtrichman and Kleinberger, 1998). The third mechanism depends on the induction of antitumoral immune response. This can be achieved via expressing toxic peptides encoded in the viral genome on the surface of the cell through antigen presenting cells triggering inflammatory cytokines as well as CTLs. These cancer-infected cells will then be recognized in the future, thus providing long-term protection against tumor recurrence.

The final mechanism of adenovirus mediating antitumor effects, which can also be considered as combined therapy, relies on arming adenovirus with therapeutic

proteins. This mechanism can be achieved via insertion of anti-cancer genes in the viral genome. Therefore, the continuous repetition of replicative cycles will result in longer transgene expression. Thus, recompensing the defect of such protein in cancer cell leading to eradicate tumor mass (Van Beusechem *et al.*, 2002). Despite all the above mechanisms, the approach of combining oncolytic adenovirus with chemotherapy is recommended for optimal oncolytic activity if it ensures limited toxicity to normal cells, which is already under clinical evaluation (reviewed in Ferguson *et al.*, 2012).

Mechanism of Antitumor Selectivity

Reaching appropriate antitumor efficacy requires viruses that are tumor selective, but the concern is how to make these viruses specific and only target tumor cells. Currently, by understanding cancer and virus microbiology, scientists have successfully developed a number of different approaches to achieve tumor selective adenovirus replication (reviewed by Mullen and Tanabe, 2002). Each approach has shown various degrees of success, however, one approach that depends on targeting defective pathways has made it not just into clinical trials but also into approval for clinical use in China in 2005 (Yu and Fang, 2007). This review will focus on this approach since it has been also used in this study with a brief highlight on the other approaches.

Targeting Tumor Receptors

Cancer cells usually express their own unique receptors on the surface of the cell, known as tumor specific receptors. One tumor selectivity approach is to redirect oncolytic adenoviruses to target only these receptors, thus facilitating selective internalization to

only tumor cells. This strategy involves the genetic modification of the viral fiber protein that is responsible for viral attachment and cell entry such as incorporation of an RGD motif in adenovirus fiber knob. However, finding these motifs has proven to be difficult. Many attempts have been made for finding motifs with high affinity of binding to receptors on the target cells, but few have successfully provided sufficient affinity (reviewed in Yamamoto and Curiel, 2010).

Targeting Tissue Specific Promoters

Adenoviruses normally express their genes under their own promoters in a wide range of cell types. One attempt to achieve tumor selectivity is to place viral replicative genes under specific promoters that restrict their expression to cancer cells rather than normal cells, which is missing the activator proteins of these promoters (Saukkonen and Hemminki, 2004). These promoters are known as cancer/tumor specific, which are found to be highly active in cancer cells. For example, the prostate-specific antigen gene (PSA), which was inserted in the viral genome upstream of the E1A sequence to control its expression in CV706 virus-infected cells. As a result, the expression of E1A protein was correlated with the expression level of this promoter in a given cell (Rodriguez *et al.*, 1997). Although this approach has provided a proof of selective viral growth, it has major drawbacks. First, it is limited by the absence of such specific promoters in many cancers. Second, tumor specific promoters are much weaker compared to viral promoters, therefore they result in attenuated E1A expression (Gu *et al.*, 2002).

Targeting Defective Pathways

Cancer cells have much in common with viruses. Both final goals are to drive cells to proliferation, which is the favor of maximum virus replication. Tumor cells are subjected to many genetic features such as loss or gain of function properties that make them fertile ground for virus replication. Fascinatingly, a number of regulatory proteins that are targeted by adenovirus for inactivation are also found to be inactivated during tumorigenesis. Thus, these viral genes become dispensable in cancer cells that have lost the target proteins. This raised the idea of engineering mutant adenoviruses that have a deletion in genes critical for viral replication in normal cells but expendable in cancer cells to eradicate them, thus obtaining a mechanism of tumor selectivity mechanism (reviewed in Morley *et al.*, 2004).

Adenoviral Vectors Targeting the Rb Pathway

In normal cells, both tumor suppressor proteins pRb and p53 are the key regulators of cancer signaling pathway that control cancer progression. In response to adenovirus infection, pRb and p53 are expressed in high levels to stop virus production. However, adenovirus inactivates this cellular defense mechanism through the expression of E1A (Whyte *et al.*, 1988) and E1B (Yew and Berk, 1992) viral proteins that counteract their function in favor of virus replication.

It is evident that tumor suppressor protein pRb is altered in many cancers due to the phosphorylation process. For example, about 30% of malignant gliomas have reported inactive Rb (Fueyo *et al.*, 1998). In addition, as it is previously mentioned, E1A viral

region has the ability to target Rb for inhibition to facilitate the entrance of the cell into S phase (Nemajerova *et al.*, 2008). Thus, a defective pRb pathway in tumor cells will provide a favorable environment for mutant virus replication, whereas the intact pathway will hinder replication. Therefore, it is suggested that deletion or mutation in the E1A viral gene will result in selective replication of E1A mutant adenovirus in pRb defective cells. Several E1A deleted adenoviruses have been engineered for oncolysis. As early as 2001, Ad-24 oncolytic adenovirus has shown promising results and is currently being tested for safety and efficacy (Suzuki *et al.*, 2001).

Adenoviral Vectors Targeting the P53 Pathway

P53 tumor suppressor protein is the most frequently altered gene in cancer. About 50% to 70% of human cancers have reported mutations in p53 gene (Petitjean *et al.*, 2007). The inactivation usually occurs through various ways including presence of mutations in the gene, and loss of p14/19^{ARF}, which functions as an indirect stabilizer of p53 protein (Zhang and Xiong, 1999). Inactivation of p53 leads to expression of dysfunctional protein resulting in a p53 defective pathway. The latter resulted in uncontrolled cellular checkpoints and failure in induction of apoptosis.

P53 is also the major target of adenovirus E1B55kDa region. Their interaction will inhibit p53 function that normally hinders virus replication (Yew and Berk, 1992; Yew *et al.*, 1994). The defective p53 pathway in most cancers gives adenovirus the green light to replicate efficiently and take over the cell cycle. Therefore, it was hypothesized that deletion of adenoviruses E1B55kDa region would result in selective replication in p53-deficient tumor cells, as E1B55kDa viral gene become superfluous in this case. Since

then, several replication selective adenoviruses have been generated. In 1996, Frank McCormick and his group engineered the first E1B deleted adenovirus (ONYX-015) that entered clinical trials to treat tumors lacking p53 function. ONYX-015 is a competent replicative adenovirus type 5 with a deletion of 827 bp in the viral E1B55kDa gene, p53-binding region (Bischoff *et al.*, 1996).

After the construction of adenovirus with E1B deletion, a series of studies were conducted to evaluate its efficacy and selectivity. One of the initial studies was performed in two cancer cell lines, RKO and H1299. The study's results reported significant reduction in virus replication efficiency in both cell lines with positive p53 status compared to the matched cell lines with negative p53 status (Harada and Berk, 1999; Rogulski *et al.*, 2000). Another study demonstrated the ability of E1B mutant adenovirus to selectively replicate in p53 mutated tumor cells and exhibited cytopathic effects (Bischoff *et al.*, 1996; Heise *et al.*, 2000). Moreover, adenovirus with E1B deletion revealed significant antitumor effect and demonstrated tumor regression in various mouse models (Rogulski *et al.*, 2000). Based on these data, human clinical trials were initiated.

Subsequently, E1B deleted adenovirus (ONYX-015) has undergone a series of clinical trials. Fifteen clinical trials from phase I to III have been conducted, spanning from 2001 to 2005, for treating 250 participants of different cancer types. Different methods of administration were tested beginning with intratumoral injection followed by other route utilized during the trials (reviewed in Kirn, 2001). E1B deleted adenovirus has shown success in the early stage with low toxicity rate and well tolerance in several trials' results including pancreatic cancer, liver tumor, prostate cancer, and ovarian cancer.

However, the available data from phase I and phase II of head and neck clinical trials have shown limited efficacy of E1B deleted adenovirus, as 50% of the cases have shown reduction in the treated tumor in combination with chemotherapy. Also, E1B deleted adenovirus as a single agent exhibited a low degree of efficacy with 13-14% complete tumor regression in another clinical setting (Ganly *et al.*, 2000; Nemunaitis *et al.*, 2001). Moreover, some *in vitro* studies have shown conflicting results, which did not come as a surprise due to the lack of consistent cell system between the studies (Rothmann *et al.*, 1998).

It has been suggested that the mechanism of selectivity of this oncolytic virus was more complex than was originally assumed. First, different mechanisms of p53 inactivation result in various degrees of viral selectivity. Second, most of the data that showed the effect of inactive p53 in supporting virus replication came from different *in vitro* cell systems and no study has been done in matched cells (Rothmann *et al.*, 1998). Third, it was suggested that deletion of E1B55kDa region might hinder its function in the exportation of viral RNA. Too soon, it became clear that tumor cells have the ability to compensate RNA export by unknown mechanism (O'Shea *et al.*, 2004). Fourth, hindering the virus spread by neutralizing antibodies that are produced from active adaptive immune response, which has been solved through the intratumoral injection of viral particles; this is most likely due to the difficulties of antibodies to penetrate tumors (Baxter *et al.*, 1994). Finally, one explanation for these contradictions has been ascribed to a lack of significant correlation between p53 genotype status and E1B deleted adenovirus replication ability (reviewed in Ganly and Singh, 2003). This indicates a need for more extensive work on identifying the role of p53 genotype in modulating E1B

deleted adenovirus replication. Also, there is a need for understanding the molecular mechanism underlying virus selectivity.

Despite all challenges above, ONYX-015 is the most utilized oncolytic virus in clinical and experimental studies and is now, since 2003, under evaluation in phase I and phase II clinical trials of many cancers (reviewed in Aghi and Martuza, 2005). In addition, FDA in China has approved H101, an oncolytic virus with the same ONYX-015 approach and an additional E3 deletion, since 2005 to treat head and neck cancers in combination with chemotherapy after the success of 4 years clinical trials (Yu and Fang, 2007). H101 is even more potent than ONYX-015 since it showed 79% overall response rate. This means that E1B deleted adenovirus is still a promising approach despite the low efficacy in some cancers, but if it is well optimized or combined with other modifications.

Tumor Suppressor P53

P53 was discovered in 1979 when it was found in a complex with viral oncoproteins SV40 T-antigen (Lane and Crawford, 1979) and later on with adenovirus E1B55K in 1982 (Sarnow *et al.*, 1982). It first came to attention when it demonstrated overexpression level in tumor cells rather than normal cells with oncogenic activity (Rotter, 1983). Ten years later, it finds its legitimate place within the family of tumor suppressor genes in the late 1980s (Finlay *et al.*, 1989).

Since p53 is a tumor suppressor protein, it provides a cellular defense mechanism against tumor formation and cancer development (Levine, 1997). It acts as a central

regulator of cell division and growth through its ability to make a decision whether genomic DNA will be repaired or if the cell will undergo apoptosis in response to DNA damage, eliminating cancer-prone cells from the replicative pool. It is therefore the key molecule to maintain the DNA integrity of the cell, thus deserved the nickname "guardian of the genome" (Lane, 1992).

P53 is localized in the nucleus, as it is a nucleoprotein that binds directly to DNA for full activation and optimal function as a transcription factor. The wild type functional protein has short half-life, 6–20 min, and is usually maintained in low levels in normal cells. If only one functional copy of p53 gene is inherited from the parents, the individual will be prone to cancer development in future. This condition in which wild type (wt) p53 allele is lost due to germline mutations is known as Li-Fraumeni syndrome (Srivastava *et al.*, 1990). However, the vast majority of tumor types result from somatic mutations in the p53 gene that led to loss of tumor suppressor function. This was clearly confirmed in studies of different tumors when each clone of ectopic, misexpressed or overexpressed, p53 cDNA has shown different sequence from the others (Piaskowski *et al.*, 2010).

P53 protein is also a crucial target for multiple viral oncoproteins that promote its inactivation as well as degradation. This includes E6 protein of human papillomavirus (HPV) as shown in cervical cancer (Scheffner *et al.*, 1990), Adenovirus E1B55kDa protein that bind to p53 protein through sequence specific mechanism (Yew and Berk, 1992), and adenovirus E1B55kDa/E4 34kDa proteins complex (Roth *et al.*, 1998).

The p53 protein belongs to a family of three members, p53, p63, and p73 proteins. They all share a homologous gene sequence for three main domains of their structures,

transactivation domain (TAD), DNA binding domain (DBD), and oligomerization domain (4D) resulting in similarities in their functions (Irwin and Kaelin, 2001). However, the p63 and p73 are not as common targets for viral oncoproteins as p53 (Roth *et al.*, 1998). Also, p73 is also not a target of mdm2 degradation as p53 (Zeng *et al.*, 1999). Although p73 can partially compensate for the p53 DBD function, mutant (mt) p53 is still able to interact with its family member through the core domain DBD, repressing their activation (Gaiddon *et al.*, 2001).

Feedback Loops

Under normal conditions p53 is tightly regulated through multiple feedback loops, positive and negative, that are initiated by p53 itself. The positive loops are essential in modulating up p53 activity in response to activation signals as well as connecting p53 activity to other signal transduction pathways. In contrast, negative feedback loops serve as p53 inhibitors, as they can modulate down p53 activity resulting in very low steady state p53 level in the normal cell (Blagosklonny, 1997).

Mouse double minute 2 (mdm2) is the main negative regulator under non-stressed conditions (Momand *et al.*, 1992). In normal condition, p53 and mdm2, an E3 ubiquitin ligase, autoregulate each other through feedback loop known as p53-mdm2 negative feedback loop to control its functionality (Lev Bar-Or *et al.*, 2000). This inactivation by mdm2 is mediated through two distinct mechanisms: one mechanism is by repressing p53 transcriptional activity through mdm2 binding to two adjacent p53 binding sites (p53BS) in the transactivation regions of p53 protein (Momand *et al.*, 1992). The other mechanism is that mdm2 and mdm4 proteins form a complex that can target p53 for degradation by

the proteasome (Parant *et al.*, 2001).

Elevated level of p53 results in increasing mdm2 levels that in turn shut off p53 activity and lower p53 level to be in a low steady state (Figure 6) (Piette *et al.*, 1997). However, under stress, both p53 and mdm2 became phosphorylated by ATM protein kinase. This phosphorylation activity blocks the binding of p53 and mdm2 proteins, thus enhancing p53 level and function (Siliciano *et al.*, 1997). On the other hand, in cancer, when p53 is mutated, it loses its ability as a transcriptional activator and therefore it cannot induce mdm2 expression, which in turn results in stabilizing p53 (Terzian *et al.*, 2008).

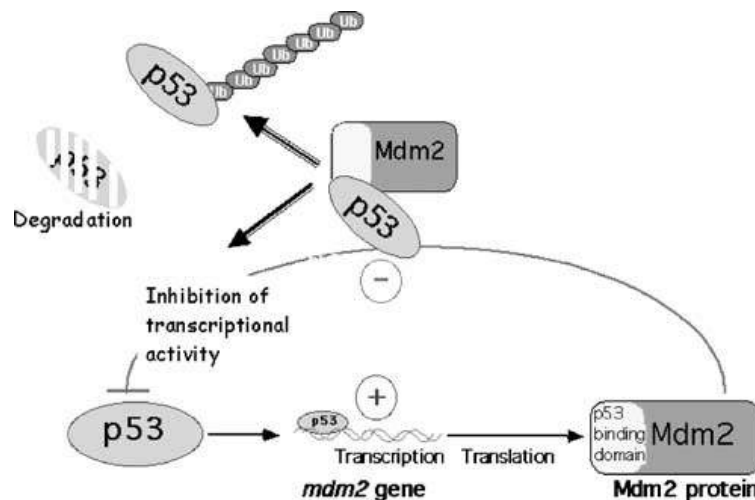


Figure 6: p53–mdm2 autoregulatory loop. Mdm2 protein binds to p53 and inactivates it through at least two distinct mechanisms: physical blockage of the transcriptional activities of p53, and promotion of p53 ubiquitination and subsequent proteasomal degradation (Reported by Oren, 2003).

P53 Protein Structure

Although p53 has complex gene structure, its sequence is conserved in all vertebrates (Jin *et al.*, 2000). Human p53 is mapped on the short arm of chromosome 17, 17p13.1 (Isobe *et al.*, 1986). It is a product of 393 amino acid protein that is translated

from one open reading frame. It consists of five highly conserved domains: the N-terminal transactivation domain (TAD), a proline rich domain (PRD), a DNA binding domain (DBD), a tetramerization domain (4D) and a C-terminal regulatory domain (Soussi *et al.*, 1990). This protein includes 12 isoforms as a result of multiple translation sites and alternative splicing sites (Bourdon *et al.*, 2005).

Transactivation Domain (TAD)

TAD is localized in the amino terminus of the protein, which is full of acidic residues and it is the target region for phosphorylation activity by kinases (Meek, 1994). Ligases and some co-activator proteins are responsible for the regulation of this domain (Mavinahalli *et al.*, 2010). It includes block I, which is highly conserved. This domain contains one of two transactivation subdomains, which is TAD1. This subdomain contains around 40 residues starting from residue 1 to residue 40 (Chang *et al.*, 1995); and more specifically two of these residues, Leu 22 (Lin *et al.*, 1994) and Trp 23 (Venot *et al.*, 1999), are critical for the transactivation activity and mutations in these residues (L22Q/W23S) would not abolish the transactivation capability.

Proline-Rich Domain (PRD)

PRD consists of about 12 proline residues and contains the second transactivation subdomain, TAD2. TAD2 is the region between residues 43 and residue 73 (Chang *et al.*, 1995). Two amino acids, Trp 53 (Zhu *et al.* 1998) and Phe 54 (Venot *et al.* 1999), are critical for transactivation activity. Mutated amino acid residues to amino acid (W53Q/F54S) can result in loss of transactivation capability. However, other single point

mutations cannot abolish the transactivation activity, but loses the ability to interact with mdm2 protein (Lin *et al.*, 1994). This means that both TADs are sufficiently stable unlike DBD, which is highly sensitive to point mutations.

In addition to transactivation activity, TAD1 and TAD2 are able to bind to multiple cellular and viral proteins through binding sites such as TATA binding protein (Chang *et al.*, 1995) and adenovirus E1B protein (Kao *et al.*, 1990), respectively. They also can interact with several regulators such as mdm2 (Kussie *et al.*, 1996). However, the amino terminal region of the transactivation domain is not specific, as it can be compensated by other regions from various transcription factors (Pietenpol *et al.*, 1994).

DNA Binding Domain (DBD)

Based on the crystallographic studies of p53 DBD, it contains a sandwich of two anti-parallel β -sheets that serve as scaffold for the domain surface, which is composed of two structural motifs with three large loops. The first motif includes loop1 that binds to DNA major groove and known as loop-sheet-helix motif. The other structural motif has two loops L2 and L3, which bind to DNA in the minor groove. Both L2 and L3 large loops can be stabilized by zinc ion (Cho *et al.*, 1994).

DBD is the most essential domain in wild type p53 protein function for several reasons. First, it contains four highly conserved blocks that are found within all p53 proteins to date (Soussi *et al.*, 1990). These blocks are found within exons 5 through 8; block ii (codons 117 to 142), block iii (codons 171 to 181), block iv (codons 234 to 258), and block v (codons 270 to 286) (Soussi, *et al.*, 1990). Second, it is the most targeted

domain for mutations in most cancer types (Caron de Fromentel and Soussi, 1992). Third, it has the binding site of one hundred of its target genes in blocks IV and V to mediate DNA binding activity and also SV40 T-antigen binding site (Tan *et al.*, 1986).

DBD mediates p53 transcriptional function of the target genes through binding to DNA in sequence-specific manner (Melero *et al.*, 2011). The p53 sequence that determines its degradation by oncoproteins is most likely located between residues 92 and 112 and it is crucial for determining p53 stability through an unknown mechanism (Gu *et al.*, 2001). DNA must have the p53-responsive element (p53RE) to facilitate the binding. This consensus sequence for p53 binding is composed of two sequences of RRRCWWGYYY separated sometimes by a variable length spacer of 0–13 nucleotides, where R is a purine, W is A or T and Y is a pyrimidine (El-Deiry *et al.*, 1992).

Tetramerization Domain (4D)

In order for wild type p53 to function properly as a transcription factor, it needs to bind to DNA as a tetramer (Melero *et al.*, 2011). This form is assembled when the protein is organized in two stably folded domains, the tetramerization and the DNA-binding domains that are linked and flanked by intrinsically disordered segments, most likely amino acid residues 323 to 355 (Melero *et al.*, 2011). Simply put, the interaction of two β -strands form a dimer and two dimers form a tetramer through the interaction with α -helices.

C-terminal Regulatory Domain

The carboxy-terminus is a highly conserved leucine-rich domain that has two

main functions: nuclear localization and as a DNA damage sensor. There are three nuclear localization signals founded in this domain. Mutations in these signals will lead to the production of cytoplasmic p53 protein (Shaulsky *et al.*, 1990). The C-terminal domain has shown its capability to form a complex with damaged DNA through the last 75 amino acid residues with a high degree of affinity to facilitate the recognition of DNA damage (Reed *et al.*, 1995). P53 structure and domains are shown in Figure 7.

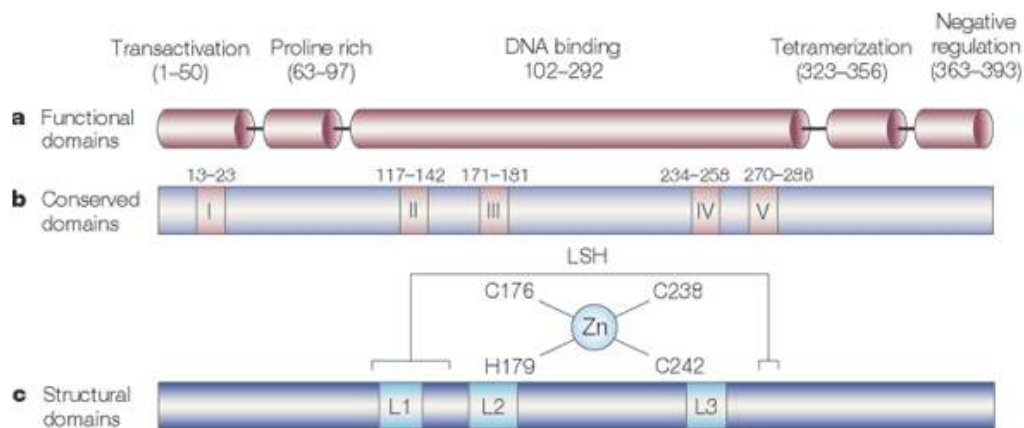


Figure 7: Schematic representation of the p53 protein. a) the functional domains. B) regions of sequence conservation, and c) structural domains. L1, L2, and L3 indicate loops, and LSH indicates a loop-sheet-helix structure (reported by Soussi and Bérout, 2001).

Function of P53 Pathway

Wild type p53 acts as a potent transcription activator of large set of responsive genes known as p53 target genes. These genes work together in a network in the p53-signaling pathway. P53 activates the expression of these genes by its binding to their promoters through its DBD. The binding occurs in a sequence specific manner via p53 (p53RE) that exists in the p53 binding sites (El-Deiry *et al.*, 1992). The target genes are involved in many biological outcomes as a result of p53 tumor suppressor activity

including cell cycle arrest, DNA repair, microRNA processing, and apoptosis (Figure 8). The latter activity is the most crucial for tumor suppression, as shown in cancer p53 based therapies (Levine, 1997).

Normally, the p53 pathway remains in standby mode until it gets activated due to stress signals that are detected by regulatory kinases. Most stress signals are associated with cancer initiation and progression including oncogenic activation (Pauklin *et al.*, 2005), hypoxia (Graeber *et al.*, 1996), and direct DNA damage such as ultraviolet (UV) irradiation (Giaccia and Kastan, 1998). When kinase enzymes detect the signals, it causes posttranslational modifications to p53 protein at different residues to promote its activation, such as phosphorylation of serine residues in N- and C-domains and acetylation at Lys370, Lys372, Lys373, Lys381, and Lys382 (reviewed in Bode and Dong, 2004).

Once p53 is activated, the p53 pathway undergoes two-step responses to be fully functional. These steps are stabilization and transcriptional activation. Firstly, the binding ability of p53 and mdm2 is diminished due to ATM kinase phosphorylation of both p53 and mdm2. This results in an accumulation of p53 protein up to 3-10 fold and stabilization for hours in the cell with the aid of multiple posttranslational modifications as well. Secondly, once stabilized, it activates the transcription of downstream target genes to induce cell cycle arrest, DNA repair, and apoptosis as final outputs, hence preventing the proliferation of cells genome damage (Kastan *et al.*, 1991; Giaccia and Kastan, 1998).

Beside the transactivation activity of p53 protein, it also has the ability to trans-

repress number of viral oncoproteins such as adenovirus E1A protein and some cellular promoters including Myc and Ras, and can also induce transcription independent apoptosis, an alternative route to cell death (Subler *et al.*, 1992; Serrano *et al.*, 1997).

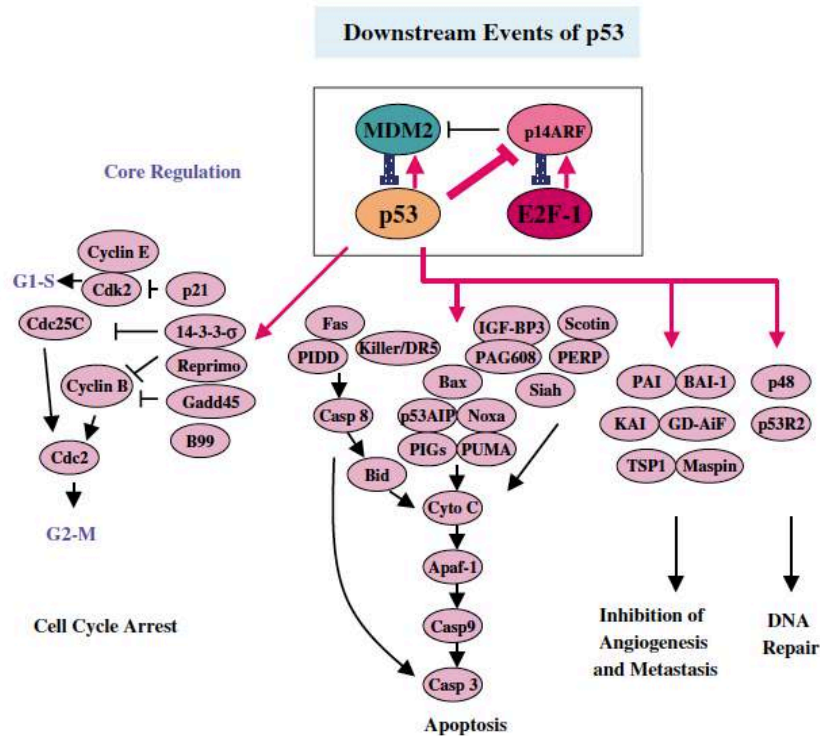


Figure 8: Downstream targets of the p53 transcription factor mediate its different biological outcomes (Reported by Harris and Levine, 2005).

Cell Cycle

The cell cycle is an ordered set of events leading to cell division. It consists of two main phases mitotic (M) and interphase. The latter contains three phases, G1, S and G2, which are the transition states for checkpoint. Normally, DNA replicates during S phase and afterwards is delivered into the two newly divided cells in the M phase. One of p53's roles in the cell division of normal cells is controlling cell cycle progression. When p53 is

activated in response to stress, it pauses the cell cycle and the progression of cell division in G1 and G2 to make the decision whether to repair the DNA damage and complete the cell cycle or to undergo apoptosis depending on the severity of the damage (reviewed in Haupt *et al.*, 2003).

Cell cycle arrest is achieved with the aid of p14^{ARF}, a p53 stabilizer. P14^{ARF} is induced as a result of the activation of E2F transcription factor (Bates *et al.*, 1998), to bind to mdm2 and inhibits its ubiquitin ligase activity and thus preventing the degradation of p53 (Honda and Yasuda, 1999). Once p53 level has risen, it transcriptionally activates a number of cell cycle genes to promote growth arrest. The genes involve Cyclin-dependent kinase inhibitor p21, 14-3-3 sigma, IGF-BP3 and BTG (El-Deiry *et al.*, 1993).

Cyclin-dependent kinase inhibitor p21 is the major player of cell cycle arrest response. Simply put, p53 protein binds to p21 protein to stimulate its expression and interaction with cdk2 cell division stimulating protein. This interaction results in the inhibition of the cdk2 kinase activity that is an essential requirement for cell cycle G1-S transition, thus stopping cell division (El-Deiry *et al.*, 1993). Moreover, p53 can also control G2-M transition via GADD45 and 14-3-3 sigma. When p53 is mutated or attenuated, it cannot function, therefore the cell divides uncontrollably and forms a tumor.

DNA Repair

P53 mediates DNA repair as a response to mild DNA damage, thereby enabling the cell to fix the damage (reviewed in Vogt Sionov and Haupt, 1999). There are a set of

genes that are involved in the DNA repair pathway, including p48 protein and p53R2, which is a ribonucleotide reductase gene that is directly involved in the p53 checkpoint for DNA damage repair (Tanaka *et al.*, 2000). Although GADD45 gene is already involved in the growth arrest outcome, it also shows a controversial role in mediating DNA repair (Kazantsev and Sancar, 1995). Once DNA repair is done, the cell cycle can resume, allowing damage-free DNA replication.

Apoptosis

Apoptosis, programmed cell death, has the most critical role in fighting cancer, which can be achieved by p53 protein. When a cell prefers to promote apoptosis rather than growth arrest and DNA repair, it means that it underwent severe damage such as viral oncogenic stress. Phosphorylation of p53 at serine46 residues by mediators such as kinases is a potent indicator of severe damage (Oda *et al.*, 2000). Upon that, p53 regulates the expression of pro-apoptotic genes in the apoptotic cascade to eliminate damaged infected cells (Kerr *et al.*, 1972).

Apoptosis involves two main pathways: extrinsic and intrinsic as shown in Figure 9. The extrinsic pathway involves the production of cell surface receptors such as Fas and DR5 proteins (Bennett *et al.*, 1998). These proteins with PIDD protein are responsible for the activation of caspase 8 and Bid, pro apoptotic protein, resulting in cytochrome c release. The latter activates the caspase, proteases cascade followed by apoptosis. On the other hand, the intrinsic pathway promotes the secretion of cytochrome c directly from the mitochondria through the expression of pro-apoptotic proteins Bax, Noxa, Puma, Bid and p53AIP1, resulting in apoptosis (Miyashita and Reed, 1995; Schuler *et al.*, 2000).

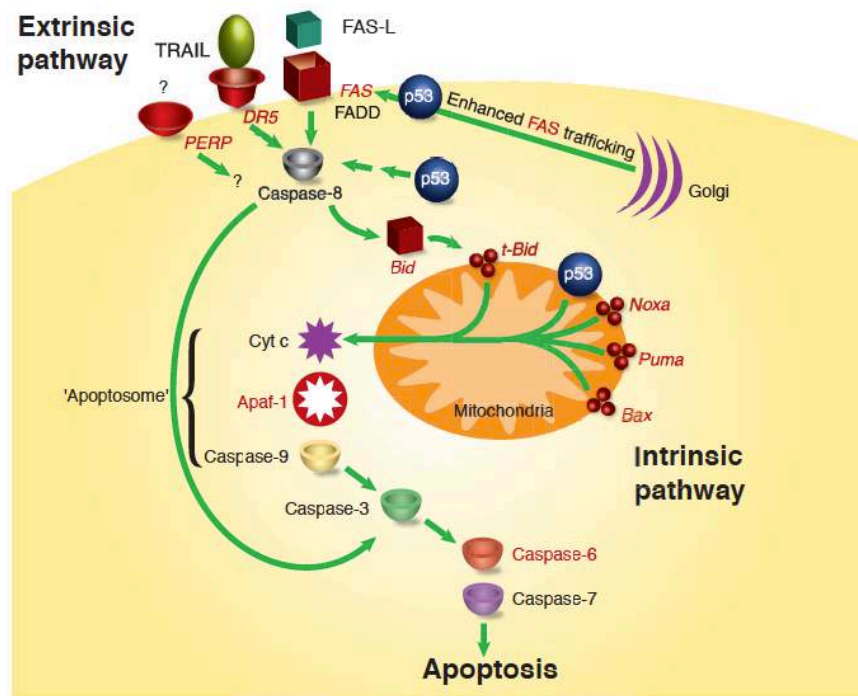


Figure 9: A model for p53-mediated apoptosis. This model depicts the involvement of p53 in the extrinsic and intrinsic apoptotic pathway. P53 target genes are shown in red. The convergence of the two pathways through Bid is shown (reported by Haupt *et al.*, 2003).

Other P53 Functions

Despite the main functions of p53, it also has other responses that contribute to safeguarding the cellular genome integrity. These responses involve other p53 inducible genes that fall into several functional categories such as differentiation, senescence, angiogenesis and metastasis inhibitors. When p53 is mutated or absent, the cell loses the ability to induce the target genes, thereby lose the capability to maintain genome stability.

Inactivation of P53

Normally, loss of p53 function results in uncontrolled cell proliferation and survival of tumor cells. Therefore, p53 plays a crucial role in carcinogenesis, since most

cancers have actively attenuated or lost p53, resulting in its stabilization in the cell. P53 inactivation and stabilization are achieved through array of mechanisms either directly or indirectly (reviewed by Vogelstein *et al.*, 2000). One of the direct mechanisms is the existence of mutations, including point mutation and small insertions/deletions (Nigro *et al.*, 1989). In contrast, one of the indirect mechanisms is by up-regulation of p53 inhibitors such as mdm2 or down-regulation of p53 stabilizer such as p14^{ARF} (Zhang and Xiong, 1999). Another mechanism is found in virus-associated cancers, as they express viral proteins that bind to p53 for inhibition and degradation such as E6 of HPV in Hela cells. Similarly, E1B55kDa and SV40 T-antigen in other cell lines (Thomas *et al.*, 1999; Wienzek *et al.*, 2000). As a consequence, mutant p53 protein loses its sequence specific binding ability and its transcription factor activity.

P53 Mutations

P53 tumor suppressor protein is damaged in 50% - 70% of cancers (Wang *et al.*, 2004a). This damage is mostly due to mutations in the gene caused by several exogenous and endogenous events, including deletion, insertion, and base substitutions, either transition (purine to purine or pyrimidine to pyrimidine) or transversion (purine to pyrimidine or vice versa) (Baker *et al.*, 1990; Petitjean *et al.*, 2007; MUTP53LOAD data base). According to the International Agency for Research on Cancer (IARC), p53-associated tumors have wide spectrum of mutations reaching around 28,717. Over 98% are high frequency somatic mutations (reviewed by Hollstein *et al.*, 1991).

P53 mutations can be divided into three distinct classes based on their impact on stabilization and DNA contact as well as structure. The three classes are: (i) class I,

missense mutation that affect protein-DNA binding; (ii) class II, missense mutations that disrupt the conformation of the protein; and (iii) class III, null mutations (deletions, insertions, and nonsense) that caused complete destruction of p53 protein (Cho *et al.*, 1994).

In fact, 95% of documented cancer associated mutations in p53 are point mutations. About 75% of them are missense that result in single amino acid changes. The latter causes alteration of the protein features and affects its functional activities as well (reviewed in Hussain and Harris, 1998). The most common missense mutations include R175H, R249S, R273H, G245S and R282W (Hernandez-Boussard *et al.*, 1999; Petitjean *et al.*, 2007).

Loss of wild type p53 transcriptional activity as a result of loss of p53 alleles is the main effect of point mutations. For complete loss, both alleles of p53 gene need to be lost or mutated, which is required for cancer initiation (Knudson *et al.*, 1975). This loss affects the transcriptional activity of DBD to various degrees because not all mutations have equal effect on p53 function. Even if they are at the same position, different amino acids have different effects. This has been confirmed in many studies, as loss of apoptotic activity of p53 due to point mutations has been reported (Chao *et al.*, 2000).

However, some mutant forms have shown novel properties that are absent in the wild type version (Frazier *et al.*, 1998). These properties are accompanied by two phenomena of different degrees of dominance (Frebourg, *et al.*, 1994). Firstly, by exerting dominant negative (DN) effect, which means that mutant p53 allele is able to inactivate the wild type p53 allele function. This inactivation involves a change in the

wild type p53 protein conformation to the mutant form, therefore loses its transcriptional activity (De Vries *et al.*, 2002). However, mutant p53 cannot be stabilized since wild type allele is still existent. This type of mutant is found in some human cancers such as Li-Fraumeni syndrome (Srivastava *et al.*, 1990).

Secondly, possessing dominant-positive effect that can be described as gain of function or acquiring new function. In this phenomenon, mutant p53 must have both alleles mutated to be able to acquire new functions, the same requirement for the loss of function (Gualberto *et al.*, 1998). Although, the mechanism of gain of function remains unclear, it results in enhanced tumorigenic potential. Several studies on some mutants have demonstrated alteration in the transcriptional activities as gain of function (Shaulian *et al.*, 1992). This type includes mutation at codons 143, 175, 248 and 281 (reviewed in Dittmer *et al.*, 1993).

There are around 280 somatic missense mutations distributed over 90 codons. Nearly 98% fall within 110 to 307 codons and mutations outlying exon 5-8 is rare (Nigro *et al.*, 1989; Petitjean *et al.*, 2007). Many of them are at hotspot codons including, 175, 248, 249, 273, 179, 157, 217, 213, 181, 195 and 280 (Figure 10). Each cancer type has different spectra of mutations and frequency as well (reviewed in Olivier *et al.*, 2010). Here, we will review the major mutations that are present in each domain, with a particular focus on the mutations that are present in high frequency in the hotspots and possess critical function as well.

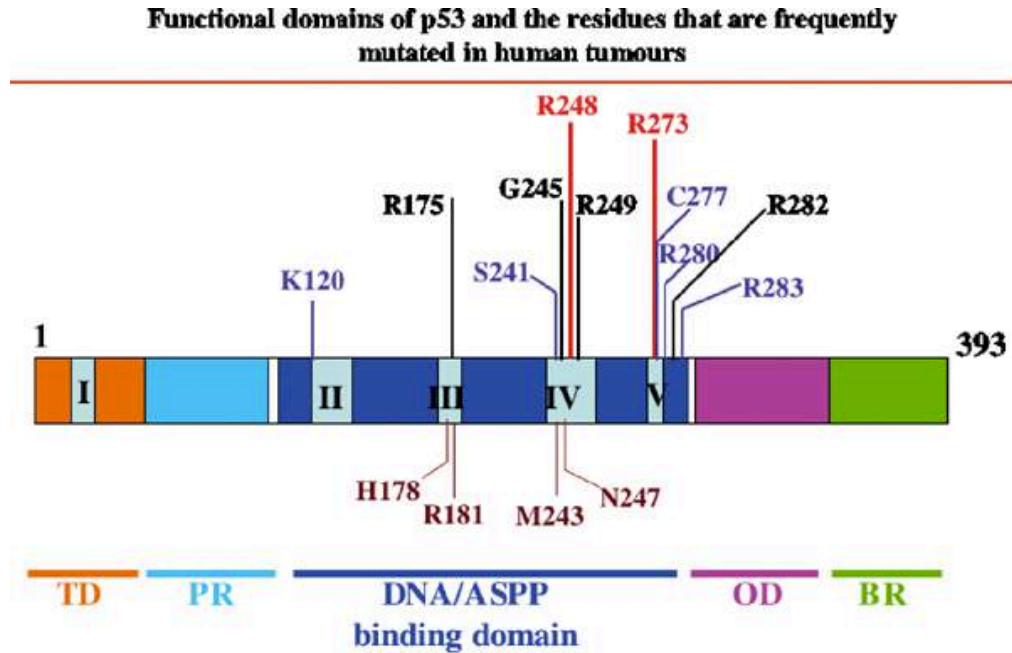


Figure 10: A diagram to show the functional domains of p53 and some of the sites that are frequently mutated in human tumours (Slee *et al.*, 2004).

P53 Mutations in the Transactivation Domain (TAD)

TAD contains multiple mutations including the first two in p53, Leu 22 and Trp 23, but these mutations mostly have no or mild alterations in p53 function (Zhu *et al.*, 1998). However, TAD contains the mdm2 interaction site, so mutations (L22Q and W23S) would cause site disruption. The latter will result in p53 stabilization and a decrease in p53 transactivation capacity (Lin *et al.*, 1994).

P53 Mutations in the Proline-Rich Domain (PRD)

Numerous single nucleotide polymorphisms (SNPs) are present at the TP53 locus. PRD is the most targeted domain for SNPs, which affect the response to therapies as well

as increase cancer development (Zhou *et al.*, 2007). PRD is also the target of several transcription binding sites such as mdm2 and p300, therefore SNPs in these binding site will prevent their interaction and abolish their function as well (Dornan *et al.*, 2003). The most common polymorphism in PRD is the variation of proline/arginine (CCC or CGC) at codon 72 (rs1042522), which results in either of two residues that are differing in their functional outcome.

Mutant p53 expressing proline allele is more efficient in transcription activation of p21 than p53 expressing arginine allele, whereas p53 arginine is a more potent inducer of apoptosis than p53 proline (Salvioli *et al.*, 2005). In addition, several studies have reported the p53 proline association with the risk of lung adenocarcinoma (Fan *et al.*, 2000), but p53 arginine mutation occurred with more frequency compared to p53 proline especially in squamous cell cancers since it is more susceptible to missense mutations than proline. Furthermore, p53 arginine showed a decreased response to chemotherapy in clinical studies. Other p53 PRD mutants and deletions have been illustrated in *in vitro* studies as they have little effects on p53 stability and function upon DNA damage, but the mutant protein remained as active as wild type p53 (Edwards *et al.*, 2003).

P53 Mutations in the DNA-Binding Domain

As previously mentioned, DBD is the main core domain in p53 protein. In fact, it is the target of more than 80% of p53 missense mutations that are clustered between amino acids 100 to 300. Each residue in this area has been found to be mutated in human tumors, and the resulting proteins display a marked heterogeneity in terms of loss of structure and function. Seven hot spot mutations, which are the most frequently mutated

in cancers, have been founded in DBD (Walker, *et al.*, 1999). Since this domain is essential in p53 main function, presence of mutations would lead to both p53 stabilization as well as loss of its transcriptional capability at various degrees such as partial activation of targets genes (Nenutil *et al.*, 2005). Depending on the nature of the mutation, they can be classified as contact mutants or structural mutants.

Contact Mutants

Mutations that are located in the protein-DNA contact region (amino acids 100–300) are referred to as contact mutants. They occurred in either the L3 loop of p53 or the nearby loop-sheet-helix motif (Cho *et al.*, 1994) and they affect the DNA-interacting residues. Some contact mutants directly interact with the DNA at the minor groove and some with the DNA phosphate backbone through the RE-p53 binding site. As a result, a protein that fails to transcribe p53-responsive genes will be produced. Mutants such as R248W, I195T, A293, N268D and R273H are examples of this type of mutation, named class I mutations. Many contact mutants were studied *in vivo* and in head and neck cancer and shown failure in the induction of cell cycle arrest and apoptosis including R248 (Erber *et al.*, 1998). A study performed by Wong *et al.* (1999) showed that contact mutants might reveal some local changes in the protein conformation and folding but in a small degree.

Structural Mutants

The other type of mutants that are not in the DNA contact region, but involved in the protein folding and conformation are termed structural mutants. They are caused by

structural perturbations within the core domain resulting in disruption of the protein folding, and thus indirectly affect DNA-binding (Cho *et al.*, 1994). They occur in the L2 loop in the zinc region leading to a local or global change of the protein conformation into an inactive form (Milner and Medcalf, 1991; Cho *et al.*, 1994). This category includes, R175H, Y220C, G245S, R249S and R282W mutations that are also known as class II mutations. R175H is the third most frequently mutated residue in cancers that has an altered conformation with intense binding to hsp70 heat shock protein.

P53 Mutations in the Tetramerization and Regulatory Domain

Although this domain is crucial for p53 activity as a tetramer, only few mutations have been documented in this area. Up to date, none of the reported mutation has shown any noticeable effect on p53 function. A study was performed on a mouse model expressing mutation S312A, which is equal to mutation S315A in humans has confirmed the previous reported statement (Lee *et al.*, 2011). Also, in response to stress, another study was conducted in fibroblasts and demonstrated no effect of this mutation on p53 function. However, multiple mutations, double or triple, in this domain may inhibit p53 activity, as shown in the mutational analysis by Waterman *et al.* (1995).

AIM OF THE STUDY

As seen in the review, there is a lack of real correlation between p53 genotype status and E1B deleted adenovirus replication ability. Therefore, the aim of this study is to understand the relationship between p53 status and the replication sensitivity of Ad5dlE1b55kDa at the molecular level. This objective was achieved by examining the following:

- 1- The effect of the quantity of wild type p53 expression and cellular backgrounds on the replication efficiency of Ad5dlE1b55kDa by evaluating the viral DNA replication ability under the expression of various levels of p53.
- 2- The effect of p53's functional activity on the sensitivity of Ad5dlE1b55kDa replication by evaluating the effect of active wild type p53 and mutant p53 on viral replication.
- 3- Assessing the functional status, transcriptional activity, of mutant p53 that supported viral growth to reveal what transcript rescued viral growth and whether this transcript lost the complete p53 function.
- 4- Determining the viral rescuing mechanism underlying selectivity and oncolysis on the basis of gene expression.

MATERIALS AND METHODS

Plasmid Construction and Cloning

Bacterial strain

Escherichia coli DH5 α was utilized in this study for cloning and propagation of plasmid DNA. This strain contains specific characteristics in its genome as follows: Δ M15, Δ (*lacZYA-argF*), U169, *recA1*, *endA1*, *hsdR17* (rK-mK+), *supE44*, *thi-1*, *phoA*, *deoR*, *gyrA96*, *relA1*, providing efficient transformation (Woodcock *et al.*, 1989).

Competent cells

Competent cells were prepared according to Inoue *et al.* (1990). The procedure was conducted as follows: a stock of cell population from New England Biolabs (NEB) was used as a source to propagate the cells. An antibiotic-free LB agar plate was utilized for growing the colonies and from the grown colonies; a single colony was used afterwards to inoculate 5 mL of antibiotic-free LB medium. After inoculation, the culture was incubated overnight in a 37°C adjusted shaker. The inoculum was then transferred into a 2L flask containing 250 mL of SOB medium. This medium consists of 0.5% (w/v) bacto-yeast extract, 2% (w/v) bacto-tryptone, 10 mM MgCl₂, 10 mM NaCl, 2.5 mM KCl, 10mM MgSO₄ and 20mM glucose. The culture was then grown by shaking at 250 rpm and 18°C until it reached OD₆₀₀= 0.6, which was measured using a spectrophotometer. Once the OD was reached, the culture was placed on ice for 10 minutes and then subjected to centrifugation for 10 minutes at 3000x g and 4°C in a Beckman GPR

centrifuge. The supernatant-free pellet was then resuspended in 80mL of pre-prepared ice-cold transformation buffer (TB: 10 mM Hepes, 55 mM MnCl₂, 15 mM CaCl₂, 250 mM KCl, at pH 6.7) and placed on ice for 10 minutes. Resuspended cells were subjected to a second centrifugation for 10 minutes at 3000x g and 4°C. The supernatant was then decanted leaving the pellet for another resuspension step in 12-15 mL of ice-cold TB. During incubation on the ice, dimethyl sulfoxide (DMSO) was added to a final concentration of 7%. The competent cells were frozen immediately in liquid nitrogen and transferred to -80°C.

Bacterial culture

Luria-Bertani broth (LB) was prepared for growing bacterial cultures. The medium contents, 0.5% (w/v) bacto-yeast extract, 1% (w/v) bacto-tryptone and 1% (w/v) NaCl were mixed together and autoclaved to sterilize the medium. The medium was kept in 50cc tubes and stored at 4°C. until further use. When the medium was used for growing cultures, the cultures were maintained at 37°C.

Luria-Bertani plates were also prepared for growing bacterial colonies by adding 2% (w/v) agar to LB medium components. For selection purposes, after autoclaving, the LB was cooled down to 45°C and 0.1 mg/mL ampicillin was added since, all plasmid constructs contain the ampicillin gene as a selection marker. Ampicillin-containing agar was poured into ten milliliter Petri dishes until it completely solidified and then stored at 4°C.

Transformation and inoculation

Ligated plasmid or plasmids isolated from bacterial cells were prepared prior transformation. One hundred microliter aliquots of frozen competent cells were used for each plasmid DNA. Cells were placed on ice and inoculated with plasmid DNA mixture. The mixture was kept on ice for 30 minutes and then subjected to heat shock at 42°C for 45 seconds. Immediately after the heat shock, the cells were placed back on the ice again for 2 minutes only. Once removed from ice, 700 µL of antibiotic-free LB was added to the mixture and incubated in the shaker for 45-50 minutes at 37°C after which 100 µL of this mixture was plated on 2X ampicillin (0.1 mg/mL) LB agar plates. This selects for growth of positively transformed colonies. After incubating the plates overnight at 37°C, the selected colonies were picked and inoculated in 2-3mL of 2X ampicillin LB and shaken overnight at 37°C.

Plasmid DNA preparation

Small-scale isolation of plasmid DNA was carried out using a commercial Kit (Plasmid DNA Miniprep Kit, Norgen Biotek Corp.), according to the manufacturer's instructions. After the isolation, the plasmid DNA was stored at -20°C for further analysis.

Large-scale isolation was also carried out for plasmid DNA using the Plasmid DNA Maxiprep Kit (Norgen Biotek Corp.), according to manufacturer's directions. The purified plasmid was then stored at -20°C and screened at a later time by restriction enzyme confirmation analysis.

Mammalian Cell Culture

Cell lines maintenance

Human Non-small-cell lung carcinoma (H1299) cells were used in this study as a p53 null cell line. They are the subclones of ATCC CRL-5803, which is derived from the metastatic site of a lymph node. Cells were maintained and cultured as a monolayer in RPMI-1640 Medium (Life Technologies, Gibco®), supplemented with 10% (v/v) fetal bovine serum (FBS, Innovative Research, Inc.) and 1% (v/v) penicillin/streptomycin (Invitrogen Corp., Gibco®). Cells were incubated in a water-jacketed incubator (Fisher Scientific, Pittsburgh PA) at 37°C with 96% relative humidity and 5% CO₂.

Cells were passaged twice weekly until they reached 90% confluency using ethylenediaminetetraacetic acid-trypsin (EDTA-trypsin) and 0.05% phenol red solution (Life Technologies, Gibco®), according to ATCC's instructions. Briefly, the medium was aspirated and cells were washed with 6 mL phosphate buffered saline (PBS at pH 7.4) (Life Technologies, Gibco®) to remove all traces of trypsin inhibitors containing-serum. Two milliliters of EDTA-Trypsin was then added to the cell monolayer of a 150 mm plate. After 2-3 minutes, cells were lifted by gently tapping the plate's sides and 4-6 mL of complete growth medium, RPMI-1640 medium, was added to stop the trypsin effect. A new culture plate was then filled with the medium and 2 mL of the cell suspension was added and cultures were incubated at 37°C.

HeLa cells, derived from cervical cancer cells of Henrietta's tumor (ATCC CCL-2) were also used in this study. The maintenance and handling of these cells was done in

Dulbecco's Minimum Essential Medium (DMEM, Invitrogen Corp., Gibco®), supplemented with 5% (v/v) fetal bovine serum (FBS, Innovative Research, Inc.) and 1% (v/v) penicillin/streptomycin (Invitrogen Corp., Gibco®). EDTA-Trypsin and PBS were used to rinse and lift the cells and two milliliters of the cell suspension was added to medium containing-culture plate. The cultured cells were then incubated at 37°C in the same incubator conditions as was stated above.

Human embryonic kidney (HEK) 293 (Microbix Inc., ATCC CRL-1573) was the third cell line used in this study. Cells were maintained in autoclavable Minimum Essential Medium (MEM, Invitrogen Corp., Gibco®) supplemented with 3% (v/v) sodium bicarbonate (Invitrogen Corp., Gibco®), 10% (v/v) fetal bovine serum (FBS, Innovative Research, Inc.), 1% (v/v) Antibiotic-Antimycotic (10,000 units/mL of penicillin, 10,000µg/mL of streptomycin, and 25µg/mL of Fungizone Antimycotic, Invitrogen Corp., Gibco®) and 1% L-glutamine (Invitrogen Corp., Gibco®). The same splitting procedure that was described in the cell lines above was carried out in HEK293 cells but with different solution. Cells were washed and lifted with saline citrate and appropriate aliquots of cell suspension were then added to new culture plate and cultured cells were then incubated at 37°C.

Cell counting

After lifting the cells from the plates, the number of cells was counted using a hemocytometer. A volume of 15µL was placed on the notch of the hemocytometer and then covered with a cover slip. The sample was visualized under the light microscope and the average number of the cells was counted in each defined field of the hemocytometer.

Based on the average number, the total number of cells in the original sample was then calculated.

Plasmid transfection using Lipofectamine 2000

The introduction of plasmid DNA into mammalian cell lines was done using Lipofectamine 2000 transfection reagent (Invitrogen Corp., Gibco®) according to the manufacturer's instructions. Briefly, when the cells were 80-90% confluent, the culture medium was replaced with antibiotic-free medium to avoid toxicity an hour prior to the transfection. For a 24-well plate, 100µL of transfection mixture (a mix of plasmid DNA and transfection reagent) was prepared for each well. A known amount of plasmid DNA was diluted in 50 µL Opti-MEM I Reduced Serum Medium (Invitrogen Corp., Gibco®). Similarly, 2 µL of Lipofectamine 2000 was diluted in 50 µL Opti-MEM I Reduced Serum Medium (Invitrogen Corp., Gibco®). Both mixtures were incubated for 5 minutes at room temperature (RT) and then mixed together and incubated again for 20 minutes at RT. The 100µL-combined mixture was then added dropwise onto the well and shaken for fair distribution. The cells were then incubated at 37°C for 5-6 hours. Following incubation, the medium was changed with complete growth medium containing an antibiotic.

Freezing and thawing

All cell lines were preserved and frozen using the same method except for the lifting step, in which HEK293 cells were lifted using saline citrate and the other cell lines used EDTA-Trypsin. When the cells reached 80-90% confluence, they were rinsed and

lifted and then centrifuged at 1200 rpm for 5 minutes at RT (Eppendorf centrifuge 5810). The supernatant was aspirated and the cell pellet was resuspended in 3 mL of freezing medium, composed of complete growth medium with 5% FBS and 10% (v/v) DMSO. Aliquots of cell suspension were then transferred to 1mL sterile cryogenic tubes (Sarstedt). The tubes were frozen immediately in dry ice and then stored at -80°C overnight. The next day, the cryovials were transferred to the liquid nitrogen tank for long-term storage.

Cell thawing was done by retrieving the vials from liquid nitrogen and pouring the content in 15 mL falcon tubes containing 5mL of the appropriate growth medium. The tubes were then centrifuged at 1200 rpm for 3 minutes at RT. The medium was aspirated and the cell pellet was resuspended in 2-3 ml of the growth medium. The cell suspension was then poured into a 150 mm cell culture plate containing 20 mL of complete growth medium and incubated at 37°C overnight. The next day, the medium was changed to remove DMSO and the cells were monitored until they became confluent.

Adenovirus production

Ad5dlE1b55kDa, is a type 5 adenovirus with a deletion in the E1B region. This was generously provided by Dr. Yousef Haj-Ahmad (Haj-Ahmad, 1986). The production of this virus was done in HEK 293 cells since it is transformed with the adenovirus E1 gene, making it a permissive cell line for adenovirus production (Graham *et al.*, 1977). Briefly, 2mL of viral inoculum was diluted in 8mL PBS⁺⁺infection medium (0.01% CaCl₂.2H₂O and 0.01% MgCl₂.6H₂O dissolved in PBS). At 90% confluency, the culture medium was aspirated and the infection medium was added to the cells and incubated at

37°C for an hour while rocking the plate. Following incubation, 20mL of growth medium was added and incubated at 37°C overnight. Cells were monitored for the cytopathic effect (CPE) for days and when they displayed complete CPE, the cells were removed with a cell scraper (BD Falcon). The cells and medium were then transferred to 50cc tube and centrifuged at 1000xg for 5 minutes. The supernatant was collected and stored at -80°C whereas the cell pellet underwent several freeze and thaw cycles to release the virus. The crude virus stock was supplemented with 10% sterile glycerol and stored at -80°C.

Ad5wt, wild type adenovirus type 5, was also provided by Dr. Yousef Haj-Ahmad (Haj-Ahmad, 1986). **AdGFP**, adenovirus type 5 with a deletion in E1 region, was kindly provided by Dr. Vanja Masic (Masic, 2013). Both virus stocks were provided in a generous amount and ready for infection of mammalian cell lines.

Adenovirus titration

Two different methods of titration were utilized to titer Ad5dlE1b55kDa: RT-qPCR and end-point titration assay according to Gustafsson *et al.*, (2012) and Ibrahim *et al.*, (2003). RT-qPCR measures the viral DNA copies per μL whereas end point dilution determines the plaque-forming unit (PFU) per mL. The end point dilution method was carried out using serial dilutions of the desired virus to infect HEK293 cells with 80-90% confluency. A ten-fold dilution of viral stock was prepared in 1 mL PBS⁺⁺. The cell monolayer of a 24-well plate was infected with 100 μL aliquot of each dilution and a growth medium was added afterwards. After the incubation period, 4 days post-infection, the displayed plaques that represent the number of viral particle were measured by

counting the area of cells in a monolayer that displayed a cytopathic effect. They appeared round and darker than other cells under the microscope. They were approximately 21 plaques. The viral concentration was determined in PFU/mL as follows: $\text{titer} = (\text{number of plaques})(\text{dilution factor})/(\text{infection volume})$.

Adenovirus infection

Adenovirus infection was carried out in 24-well plates by using a viral volume equivalent to the desired multiplicity of infection (MOI). This volume was then mixed with PBS⁺⁺ in a total volume of 100 μL /well. Then, it was added to the cell monolayer after aspirating the medium. The plate was then incubated for an hour, with swirling every 15 minutes, at 37°C in an incubator using 5%CO₂ and 96% humidity. Two milliliters of growth medium was then added to each well and the plate was maintained in the incubator.

DNA/RNA Manipulation

DNA isolation

Total DNA and total RNA were isolated from the same sample by using the RNA/DNA/Protein Purification Kit (Norgen Biotek Corp.), according to the manufacturer's instructions. The purified DNA and RNA were examined for quality by agarose gel electrophoresis.

DNA quantification

Total DNA samples were quantified using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific) in which the absorbance was measured at wavelengths 260 nm (A_{260}) and 280 nm (A_{280}). According to Glasel (1995), the value of A_{260}/A_{280} ratio was used as determinant of sample purity. A sample's ratio value between 1.8 and 2.0 is considered pure.

Cleaning of PCR product

The amplified fragments that were obtained from a PCR reaction were cleaned using the PCR Purification Kit (Norgen Biotek Corp.), according to the manufacturer's instructions. Briefly, the amplified DNA product was subjected to clean up and purification step, which is based on spin column chromatography that bind DNA in an ionic concentration manner, to remove PCR by-products. The purified samples were then used as inserts for the subsequent step of cloning.

Restriction enzymes digestion

Multiple digestions with restriction enzymes were carried out for downstream cloning purposes. The restriction enzymes were obtained from New England Biolabs (NEB) and used according to the manufacturer's directions. Restriction enzyme digestion was used for either digesting the fragments of vectors and inserts for the ligation step or in the confirmation of the desired clone. In digestion preparations used for confirmation purposes, 2 to 5 μ g of DNA was digested using one to ten units of restriction enzyme; 2 μ L provided buffer and Nuclease-free water was added to complete the 20 μ L reaction

volume. This reaction was then incubated for two hours followed by agarose gel electrophoresis.

However, in digestion preparations used for obtaining vectors and inserts fragments, larger volumes and longer incubation times were performed according to NEB's protocol. In this digestion type, multiple enzymes were required, equal amount of enzymes were used and the reaction was done in the most suitable buffers for optimal enzyme activity. In the case of incomplete digestion, the amount of DNA and enzymes were adjusted. Also, a sequential digestion was conducted to ensure the activity of the enzymes and to avoid incompatible enzymes. In addition, enzyme inactivation at 70°C for 20 minutes was performed when required.

DNA ligation

The digested and cleaned fragments of vectors and inserts were then ligated in a 20µL reaction volume. One microliter of T4 DNA ligase (New England Biolabs) that contains 400 units was used for this purpose, according to the manufacturer's instructions. The reaction was done at RT for 2-3 hours to ligate the sticky ends of the two DNA fragments. However, when ligating blunt ends, reactions took 8 hours at RT or occasionally overnight at 16°C.

Synthetic oligonucleotides annealing

Two oligonucleotides were used for the construction of poly-A signal and p53R72P fragments. All were purchased from Integrated DNA Technology (IDT) flanked with restriction enzyme sites for cloning purposes. The fragments were mixed and

annealed through the use of the iCycler PCR machine (Bio-Rad). The PCR program used was as follows: the mixed oligonucleotides were heated to 94°C and the temperature was lowered in increments of 0.5°C every 10 seconds until it reached 18°C and with a final cooling down to 4°C.

Oligonucleotide ligation

The two annealed oligonucleotides were then ligated together in a 20µL reaction volume containing 1X *Taq* DNA ligase buffer and using 40 units of *Taq* DNA ligase (New England Biolabs), according to the manufacturer's instructions. The reaction was then incubated at 45°C for 15 minutes. The ligation process was dependent on forming a phosphodiester bond between 5`phosphate and 3` hydroxyl end of the two annealed oligonucleotides.

DNA sequencing

DNA sequencing of all cloning steps starting from the PCR product continuing until the desired constructs was done using Applied Biosystem 3130x Genetic Bioanalyzer DNA sequencer. This instrument can provide real time detection of DNA sequences using dideoxy DNA sequencing method that was developed by Sanger *et al.* (1977). Cycle sequencing reaction was carried out along with BigDye Terminator chemistry using the BigDye Terminator v1.1 Sequencing Standard Kit (Applied Biosystems). Multiple forward and reverse primers (Table 1) that are specific for each construct were utilized to amplify 800-1000 long fragments. These data were directly

analyzed for their sequence as well as mutation detection using Finch TV software as base calling program.

Table 1: List of sequencing primers used in this study

Name	Sequence
P72-F	5`CAGACCTATGGAACTACTTCC3`
P175-F	5`ACTGAAGACCCAGGTCCAGATGAAG3`
P175-R	5`AGGGGCCGCCGGTGTAGGAGCTGC3`
P53-F	5`TCCCTTCCCAGAAAACCTACC3`
P53-R	5`CTAGATGGCGCCTCAGTCTGAGTCAGGCCCTTC3`
P268-F	5`CGCCTGAGGTTGGCTCTGACTGTACCACCAT3`
MARI-F	5`TCCCCGGGTTAGTAAGACATCACCTTGCATT3`
MARI-R	5`TGCTCGAGAGCCATAGTTTGAGTTACCCTTT3`
HPRE-F	5`CTAGATGGCGCCTTGCTCGGCAACGGCC3`
BGH-F	5`TCGAATTCTGTGCCTTCTAGTTGCCAGC3`

Total RNA isolation

The isolation of total RNA was carried out using the Total RNA Purification Kit (Norgen Biotek Corp.), according to the manufacturer's directions.

RNA quantification

All purified total RNA samples were quantified using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific). The quantification of RNA sample was dependent on measuring the absorbance at wavelengths 260 nm (A_{260}) and 280 nm (A_{280}) (Glaser, 1995).

Analysis of RNA on Agilent 2100 Bioanalyzer

RNA samples were analyzed for their quality using the Agilent RNA 6000 kit with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.), according to the manufacturer's instructions. Simply, Bioanalyzer uses a lab on a chip approach to

determine RNA integrity using the same principles as agarose gel electrophoresis.

DNase treatment of RNA samples

For pure isolated RNA samples, the residual DNA in the RNA sample was digested using TURBO DNase (Ambion). In a total reaction volume of 100 μ L, 50 μ L of RNA sample was mixed with the provided buffer and four units of TURBO DNase. The prepared reaction was then incubated at 37°C for 30 minutes.

RNA cleaning

The cleaning of RNA samples was done using the RNA CleanUp and Concentration Kit (Norgen Biotek Corp.), according to the manufacturer's instructions.

DNA/RNA Agarose Gels

Agarose gels for DNA samples

Agarose gels were made according to the procedure of Sambrook *et al.*, (1989). DNA samples were run on various percentage agarose gels depending on the size of the fragment to be analyzed and the amount of DNA in the sample. Agarose gels were then visualized under ultraviolet (UV) light. The pictures were then captured using the AlphaImager 2200 (Alpha Innotech).

Agarose gel extraction

The extraction of DNA fragments for cloning, either vector or insert, after running on agarose gel was carried out using the DNA Gel Extraction Kit (Norgen Biotek Corp.), according to the manufacturer's directions.

Formaldehyde agarose gels for RNA samples

The integrity of isolated and cleaned RNA was determined using formaldehyde agarose gel electrophoresis. The gel was prepared according to Sambrook *et al.* (1989). Gels were then exposed to UV light and pictures were captured using the AlphaImager 2200 (Alpha Innotech).

PCR

Polymerase chain reaction (PCR) (Mullis *et al.*, 1986) was performed according to the manufacturer's instructions of the polymerase. All of the forward and reverse primers (Table 2) that were used in PCR reactions were carefully designed and also ordered through the IDT. These primers were utilized at a concentration of 50 μ M by diluting them in Nuclease-free water (Ambion) and then stored as aliquots at -20°C.

All end point PCR reactions were performed in a Bio-Rad iCycler thermal cycler. A volume of 20 μ L was prepared for each reaction consisting of 1-2 μ L of DNA or complementary DNA (cDNA) template, 0.12 μ L of each primer (50 μ M stock), 1 μ L deoxynucleotide triphosphates (dNTPs) (10mM), 2 μ L of the supplied 10X reaction buffer, 0.2 μ L (2.5 units/ μ L) of *pfu* polymerase or *Taq* DNA polymerase (5 units/ μ L),

and the reaction volume was completed with distilled water (dH₂O). The PCR reaction program was as follows: 94°C for 3 minutes for template denaturation. Next, annealing was done for 15 seconds. The applied annealing temperature was variable, but it was 5°C lower than melting temperature (T_m). Following that, extension at 72°C for 1/2 minute per 1kb and a denaturation at 94°C for 30 seconds. The number of cycles was between thirty and forty cycles finishing with a hold for 5 minutes at 72°C and a final incubation at 4°C.

Real-time PCR

Probe-based qPCR reactions were performed in the CFX Connect Real-time PCR Detection System machine (Bio-Rad) to evaluate the gene expression level of p53 as well as viral DNA level. For the reverse transcriptase reaction, known concentrations of DNA/cDNA in which the initial RNA volume was equivalent to 200-300 ng were used. Specific probes for the p53 gene, adenoviral hexon gene and housekeeping genes along with specific primers were utilized to amplify the desired region (Table 2&3). In addition, plasmid copy number was determined through the use of a standard curve of known plasmid concentration. The reaction mixture contained 3µL of cDNA/DNA template, 10 µL of 2X PCR master mix (Norgen Biotek Corp.) 2 µL of primer mix (5µM stock), 1µL of specific probe for each gene (10µM) in a 20µL reaction. The TaqMan qPCR program was performed on the reactions as follows: the mixture was heated to 95°C for 3 minutes followed by denaturation at 95°C for 15 seconds. Then, the reaction underwent annealing followed by extension for 30 seconds at 60°C. The last cycle was repeated for 40 cycles.

On the other hand, SYBR GREEN-based Real-time PCR using CFX Connect

Real-time PCR Detection System machine (Bio-Rad) was carried out to evaluate the gene expression of p53 target genes. Specific forward and reverse primers for each gene were used (Table 2). The reaction contained 7.5 μ L of 2X SYBR GREEN master mix (Bio-Rad), 0.12 μ L of each primer (50 μ M stock) and 3 μ L of cDNA/DNA template in 15 μ L reaction. The mixture was heated to 94°C for 15 minutes for the activation of the hotstart enzyme. The cycling reaction was performed as follows: the temperature was set at 95°C for 15 seconds, then 30 seconds for annealing at a temperature that is 5°C lower than the primer melting temperature and then reduced to 72°C for one minute. This was repeated for 40 cycles. Following this, the temperature returned to the annealing temperature for 1 minute and the melting curve was carried out by lowering the temperature by 0.5°C increments every 10 seconds for 80 rounds.

Table 2: List of primers and oligonucleotides used in this study

Name	Sequence
P53pro-F	5'CTAGATGACGTCCCTAGGAGATCTCGAGGGGAGAAAACGTTAGGGTGTG3'
P53pro-R	5'GTCGAGCATATGGGCAGTGACCCGGAAGGCAG3'
TP53-F	5'CTAGATCATATGGTCTAGAGCCACCGTCCAGG3'
TP53-R	5'CTAGATGGCGCCTCAGTCTGAGTCAGGCCCTTC3'
MARI-F	5'TCCCCGGGTTAGTAAGACATCACCTTGCATTT3'
MARI-R	5'AACAGCCATAGTTTGAGTTACCCTTT3'
MARII-F	5'TGCCTAGGTTAGTAAGACATCACCTTGCATTT3'
MARII-R	5'TGCTCGAGAGCCATAGTTTGAGTTACCCTTT3'
HPRE-F	5'CTAGATGGCGCCTTGCTCGGCAACGGCC3'
HPRE-R	5'CTGAATTCGACATTGCTGGGAGTCCAAGAG3'
BGHpolyA-F	5'TCGAATTCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTG CCTTCCTTGACCCTGGAAGGTGCCACTCCCCTGTCCTTTCCTAATAAAATGAG GAAATTGCAT3'
BGHpolyA-R	5'TCCCCGGGGCCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCCCACC CCCCAGAATAGAATGACACCTACTCAGACAATGCGATGCAATTTCTCATTTTA TTAGGA3'
PUC19-F	5'GCAGAAAAAAGGATCTCAAGAAG
PUC19-R	5'TAACTGTCAGACCCAGTTTACTC3'
Adeno-F	5'CGGGTAATATGGGTGTTCTGG3'
Adeno-R	5'GCTCTGTGTTTCTGTCTTGC3'
GAPDH-F	5'AAATTCAACCTCTTGGGCCCTCCT3'
GAPDH-R	5'AGGCGCCCAATACGACCAAATCTA3'
S15-F	5'CTACAACGGCAAGACCTTCA3'

S15-R	5` GGCTTGTAGGTGATGGAGAAC 3`
Fas-F	5` AAGTGACTGACATCAACTCC3`
Fas-R	5` CACTTCTAAGCCATGTCC3`
BAX-F	5` AGGGTTTCATCCAGGATCGAGCAG3`
BAX-R	5` ATCTTCTTCCAGATGGTGAGCGAG3`
MYC-F	5` TACCCTCTCAACGACAGCAG3`
MYC-R	5` TCTTGACATTCTCCTCGGTG3`
ZEB2-F	5` GGCGCAAACAAGCCAATCCCA3`
ZEB2-R	5` TTCACTGGACCATCTACAGAGGCTT3`
PRB53-F	5` TAACAGTTCCTGCATGGGCGGC3`
PRB53-R	5` AGGACAGGCACAAACACGCACC3`
Mega-F	5` GACTTGACGCTACTCCCCTGC3`
Mega-R	5` TAAGATGCTGAGGAGGGGCCAG3`
E1B-F	5` GAATGAATGTTGTACAGGTGGCT3`
E1B-R	5` AGGAAAACCGTACC GCTAAAATTG3`
BGH-F	5` TCGAATTCTGTGCCTTCTAGTTGCCAGC3`
BGH-R	5` TCCCCGGGGCCTGCTATTGTCTTCCCA3`
5S-F	5` GCCATACCACCCTGAACG3`
5S-R	5` AGCCTACAGCACCCGGTATT3`
ATM-F	5` TGAAGAAGGAAGCCAGAGTACA3`
ATM-R	5` CCCACATTGCTTCGTGTTTC3`
CASP2-F	5` CAAAGTGGGCAGTTTCAGCC3`
CASP2-R	5` ACATATCCTCCAGGTGGCCT3`
ATR-F	5` ACACTGAGAACTGGCCTTCG3`
ATR-R	5` ATGATCCAGGCAGCGAACAA3`
CHEK-F	5` TTTGCCAATCTTGAATGTGTGA3`
CHEK-R	5` AGGTTCCATTGCCACTGTGAT3`
MDM2-F	5` AGGAGATTTGTTTGCGTGC3`
MDM2-R	5` TGAGTCCGATGATTCCTGCTG3`

Table 3: List of probes used in this study

Probe Name	Sequence
P53	5`/56-FAM/CGGAGGCCCC/ZEN/ATCCTCACCATCATCA/3IABkFQ/3`
GAPDH	5`FAM-AGGAGATGC/ZEN/TGCATTGCCCCCTTAA/3IABkFQ3`
Adeno.	5`FAM-CATTCAACT/ZEN/GCGATGCTTGGCCC/IABkFQ3`
5S	5`/56-FAM/TT AGT ACT T/ZEN/G GAT GGG AGA CCG CCT /3IABkFQ3`
S15	5` /56-FAM/AGG TGG AGA /ZEN/TCA AGC CCG AGA TGA /3IABkFQ/3`

Site directed mutagenesis PCR

The introduction of mutations in the p53 wild type sequence was achieved through the use of mega primer strategy that was developed by Tyagi *et al.* (2004). In this strategy, two rounds of PCRs were conducted using two intact flanking primers and one mutagenic primer to generate the required fragment. Since one of the constructed plasmid

(pHPRE) contains the p53 wild type sequence, it was used as a template of the first PCR reaction to amplify the target region. In the first round, two primers (forward and reverse), of which one of them is mutated, were used to generate the first PCR product that contains the desired mutation. Then, the first product was used as a template for the second round of PCR reaction using intact forward and reverse primers to amplify the mutant fragment thus generating the final mutant p53 PCR fragment. This was then used in subsequent cloning steps. Primers are listed in Table 4.

Table 4: List of mutated primers used in this study

Name	Sequence
72R-F	5'AGGTCCAGATGAAGCTCCAGAAATGCCAGAGGCTGCTCCC CG GTGGCC CCTGCAC CAGCAGCTCCTACA3`
72R-R	5'CCGCCGGTGTAGGAGCTGCTGGTGCAGGGGCCAC CG GGGAGCAGCCTCTGGCATTCT GGGAGCTTCATCTGGACCTGGGTCTTCAG3'
175H-F	5'TGCACGTACTCCCCTGCCCTCAACAAGATGTTTTGC3`
175H-R	5`GATGCTGAGGAGGGGCCAGACCATCGCTATCTGAGCA GTG CTCATGGTG3`

Reverse transcription PCR

Reverse transcription PCR was performed on RNA samples to generate cDNAs that were used as template for end point PCR and qPCR reactions. The mixture contained 300-500 ng of total RNA mixed with 0.5µL of 100mM oligo (dT)₁₈ primer (IDT) and the reaction volume was then brought up with RNase/DNase-free water to 5µL. The reaction program was as follows: the mixture was heated to 70°C for 5 minutes and then held at 4°C. During the hold, 15µL of RT reaction solution was added to the mixture. This solution contained 4µL of 5X First Strand Buffer (250mM Tris-HCl [pH 8.3 at RT], 375mM KCl, 15 mM MgCl₂), 1µL of 10mM dNTPs, 2µL of 0.1 M dithiothreitol (DTT),

0.2µL Superscript III reverse transcriptase (Invitrogen) and 7.8 µL RNase/DNase-free water (Ambion). The synthesis of cDNA was then continued at 25°C for 5 minutes, 42°C for 90 min and 70°C for 15 minutes followed by maintaining the temperature at 4°C.

Gene expression profiling by microarray

The transcriptome expression analysis was performed on selected RNA samples using Affymetrix Genechip Human transcriptome array 2.0 (HTA 2.0) (Affymetrix, CA, USA) at Sick Kids Hospital, (Genomic Facility, Toronto, Ontario). The GeneChip system is comprised of 25-mer oligonucleotide probes synthesized on quartz chips and assembled into cartridges. Hybridization and scanning was performed according to the manufacturer's instructions (Affymetrix, CA, USA).

Data analysis

All statistical analysis was performed in Excel (Microsoft Corp., USA). Two tests, the analysis of variance one-way ANOVA and the Student t test, were utilized in this study to calculate the level of significance. P values <0.05 were considered significant.

RESULTS

ENGINEERING AND EVALUATING P53 EXPRESSION VECTORS IN MAMMALIAN CELLS

Efficient gene expression requires a vector that remains in the cell for a prolonged period of time. Therefore, a plasmid DNA (pDNA) vector was designed to maximize survival and to enable high p53 expression level. There are a number of DNA and RNA cis-acting regulatory elements that can increase plasmid stability and stabilize transgene expression *in vitro*. These elements include matrix attachment regions (MARs) that are able to enhance and maintain transgene expression over an extended period of time as well as enhancing pDNA stability (Jenke *et al.*, 2002; Allen *et al.*, 2000; Wang *et al.*, 2008), hepatitis B virus posttranscriptional regulatory element (HPRE) that promotes RNA accumulation and thus enhancing transgene expression (Huang and Liang, 1993; Huang and Yen, 1994, 1995; Huang *et al.*, 1996; Smith *et al.*, 1998) and bovine growth hormone poly-A signal (BGH poly-A) that increases plasmid resistance as a result of a reduction in enzyme activity and enhanced mRNA posttranscriptional efficiency (Pfarr, 1986; Azzoni *et al.*, 2007). These elements were utilized to construct various transient p53 expression vectors. These vectors were then evaluated for their stability *in vitro* in three different cell lines, H1299, HEK293, and HeLa.

Plasmid construction

To construct various p53 expression vectors that contain the transgene and all the regulatory elements, six plasmids were engineered through a series of cloning steps and strategies. All vectors are based on the PUC19 backbone. The first expression vector was engineered to contain the p53 promoter only (p0). The second plasmid contained the p53 promoter in addition to a p53 transgene (pA). Another four plasmids were constructed as follows, pB (contains p53 promoter, p53 transgene and MAR element), pC (contains p53 promoter, p53 transgene and two copies of MAR elements, one is upstream the transgene and one is downstream). pD (contains p53 promoter, p53 transgene, two copies of MAR elements and HPRE) and pE (contains all above components in addition to BGH poly-A signal). The cloning strategy and final engineered plasmids are shown in Figure 11. Furthermore, restriction enzyme digestion confirmation of constructed plasmids was done and the expected bands were observed on the gel as shown in Figures 12, 13 and 14.

Table 5: Engineered p53 expression vectors in this study

Plasmid name	Contents	Figures	Plasmid abbreviation used in transfection
P0	P53 promoter	Figure 12	-
PA	P53 promoter + P53 gene	Figure 12	A
PB	P53 promoter + P53 gene + MARI	Figure 13	B
PC	P53 promoter + P53 gene + MARI + MARII	Figure 13	C
PD	P53 promoter + P53 gene + MARI + MARII + HPRE	Figure 14	D
PE	P53 promoter + P53 gene + MARI + HPRE + MARII + Poly-A	Figure 14	E
PF	Negative control	-	F

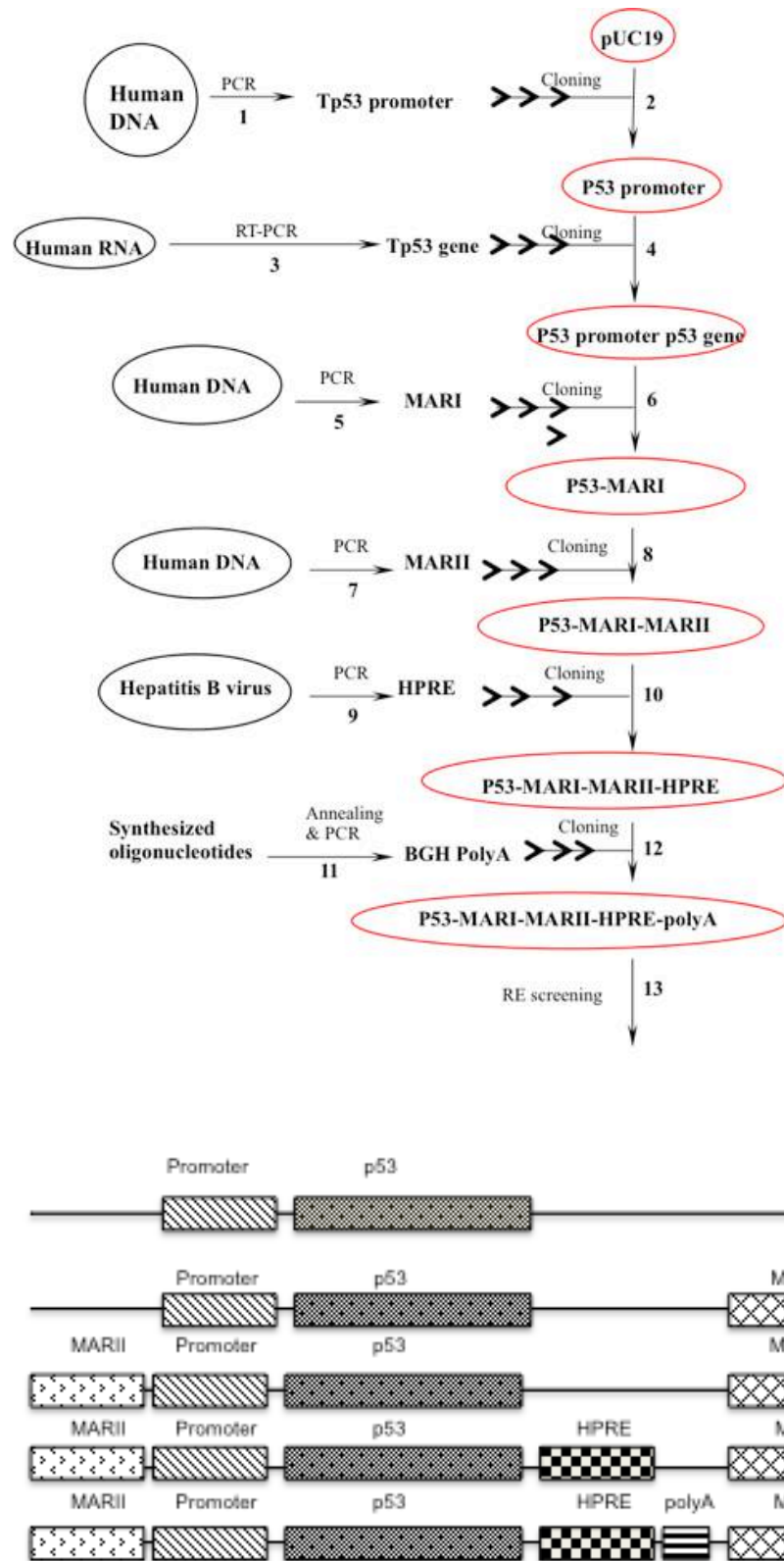


Figure 11: Design scheme used for the construction of the various p53 expression vectors

Table 6: Restriction enzyme analysis of p53 expression vectors (P0 and PA)

Plasmids	Enzyme	Expected band size (bp)
P53 promoter (P0)	<i>ZraI/NdeI</i>	2435/543
P53 promoter p53 gene (PA)	<i>NdeI/EcoRI</i>	2766/1483

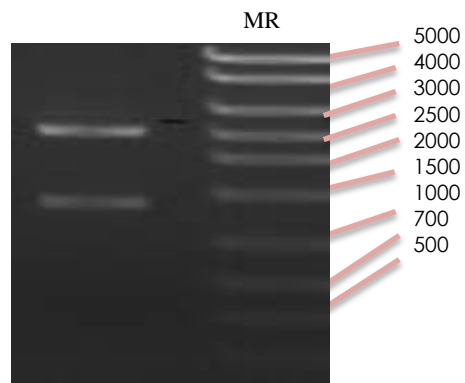
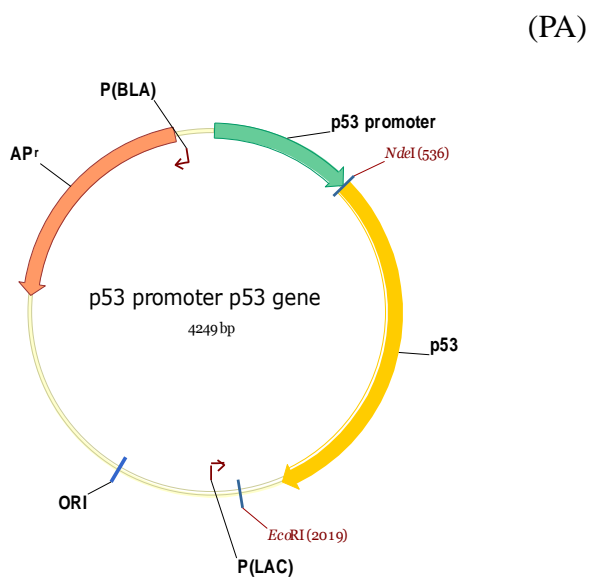
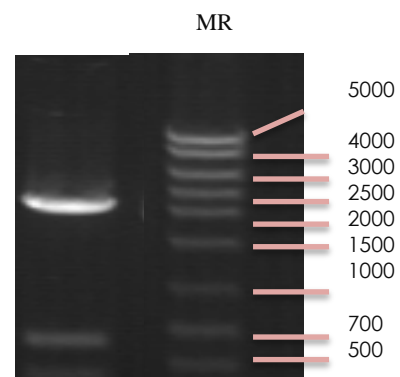
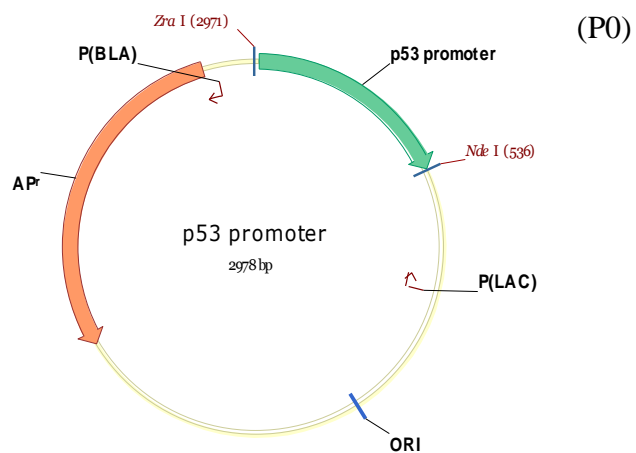


Figure 12: Schematic diagrams of p53 expression vectors (P0 and PA) and confirmation of test plasmids by restriction enzymes digestion. MR: Norgen's MidRanger ladder.

Table 7: Restriction enzyme analysis of p53 expression vectors (PB and PC)

Plasmids	Enzyme	Expected band size (bp)
P53-MARI (PB)	<i>PstI/NsiI</i>	4688/330
P53-MARI-MARII (PC)	<i>NdeI/EcoRI</i>	4489/1862

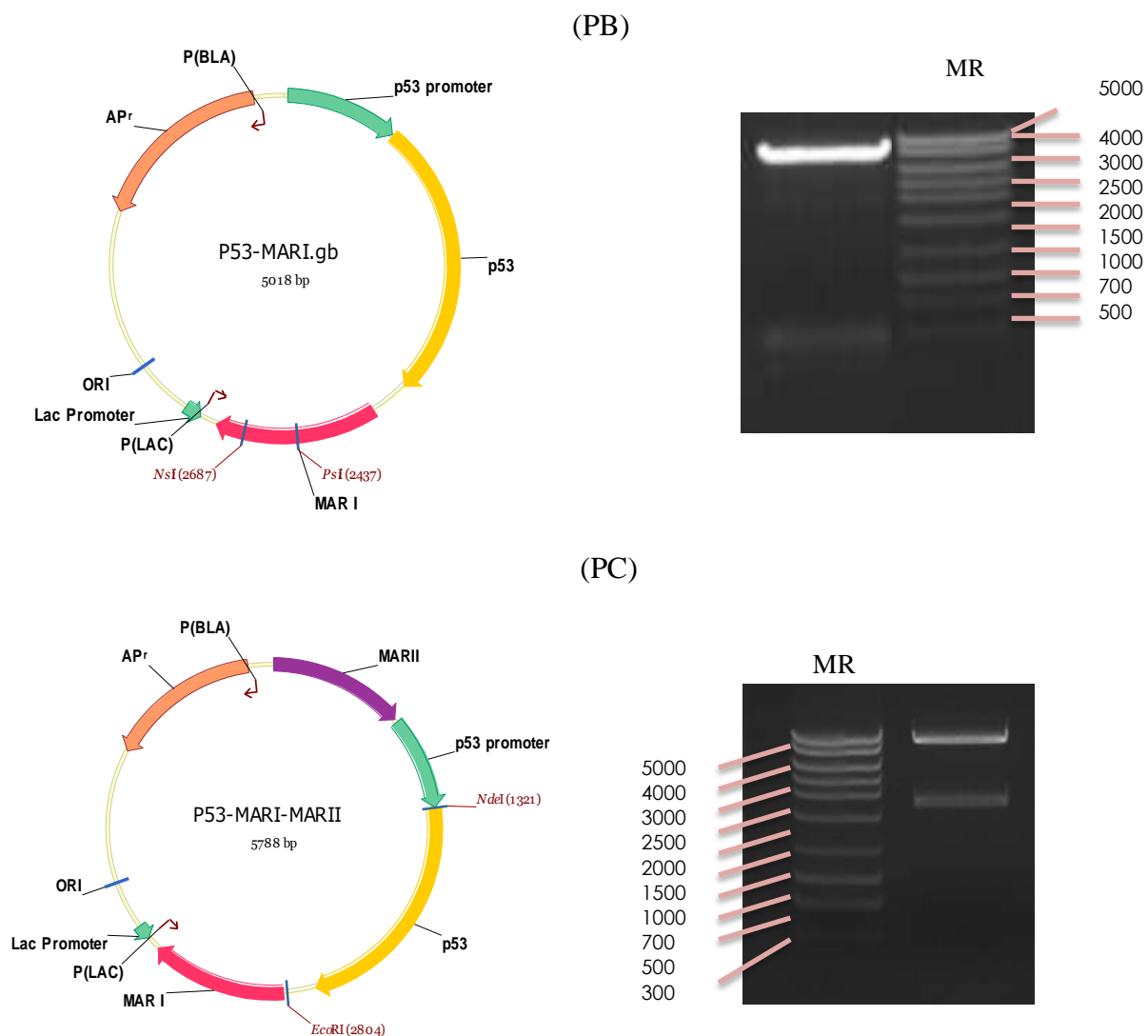


Figure 13: Schematic diagrams of p53 expression vectors (PB and PC) and confirmation of test plasmids by restriction enzymes digestion. MR: Norgen's MidRanger ladder.

Table 8: Restriction enzyme analysis of p53 expression vectors (PD and PE)

Plasmids	Enzyme	Expected band size (bp)
P53-MARI-MARII-HPRE (PD)	<i>Bam</i> HI	4129/2038
P53-MARI-MARII-HPRE-PolyA (PE)	<i>Bam</i> HI	4317/2038

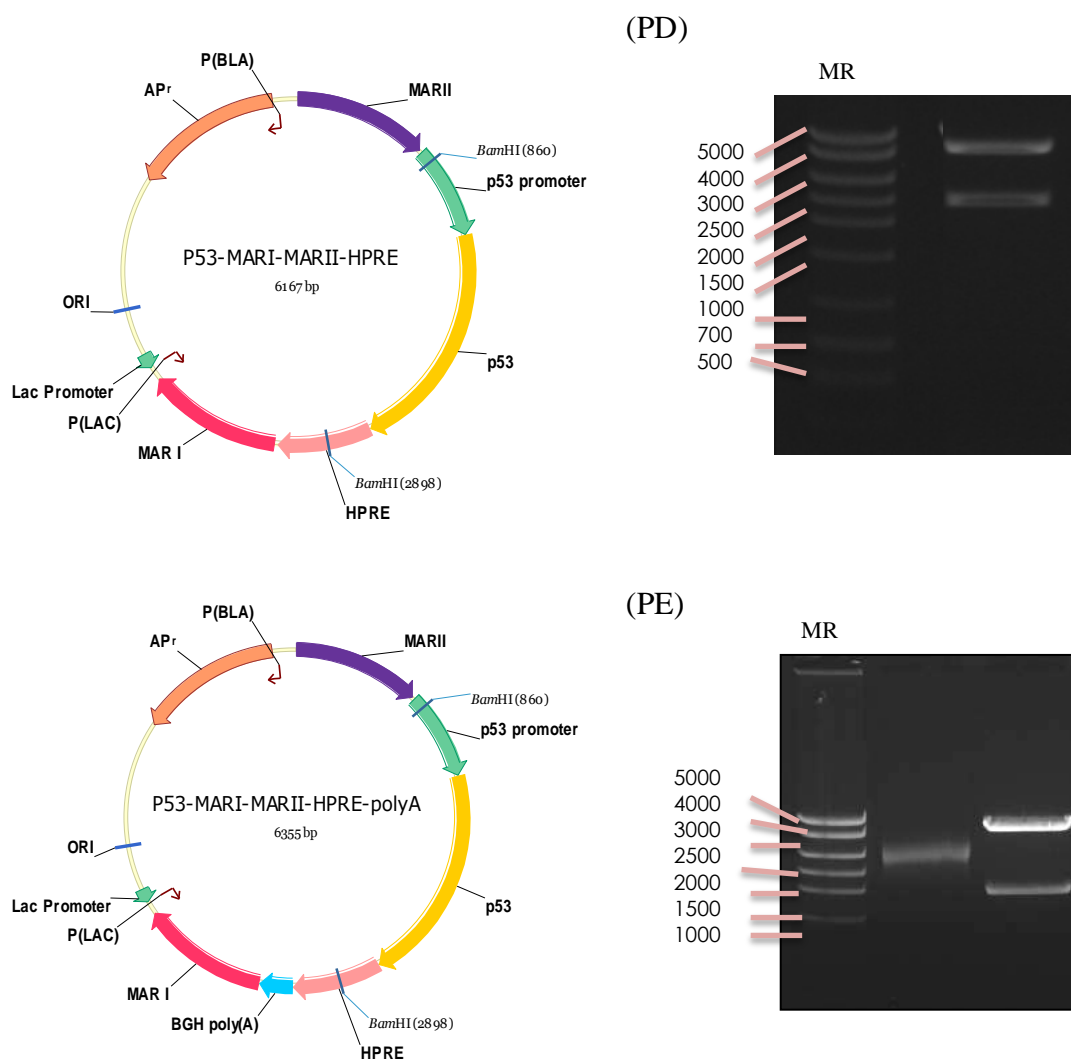


Figure 14: Schematic diagrams of p53 expression vectors (PD and PE) and confirmation of test plasmids by restriction enzymes digestion. MR: Norgen's MidRanger ladder.

Evaluation of p53 expression vector stability in H1299, HEK293, and HeLa cell lines

The impact on p53 transgene expression was evaluated by determining the plasmid stability as well as p53 mRNA level over time. Plasmids A, B, C, D, E and F as shown in Table 5 were used in this experiment. In order to assess their stability over time, equal copy number from each of the plasmids was transfected into three cell lines H1299, HEK293, and HeLa. Transfection was conducted in triplicate and cultured in a 24-well plate using cationic liposome transfection reagent (Lipofectamine 2000) and the medium was replaced 6 hours post-transfection. Following transfection, samples were collected at various time points dependent on the cellular growth rate of each cell line. Total DNA and RNA were isolated, quantified and processed for further analysis. The data were analyzed using a one-way ANOVA test as well as a Student t test. P values <0.05 were considered significant.

Plasmid stability in H1299 cells

Equal amounts of DNA from each point (day 1, day 4, day 8 and day 13) were used as templates for qPCR to assess plasmid stability. The actual plasmid copies per cell were obtained from a standard curve of known plasmid concentration using specific primers for p53 gene since this cell line is p53-null. From the data illustrated in Figure 15, plasmid copy numbers were shown to reach the highest point at day 1 post-transfection and they decreased sharply at day 8 post-transfection, reaching the basal level at day 13 post-transfection. Plasmid D revealed the highest copy number (5 copies per cell) at day 13 post-transfection compared to test and negative control plasmids (one-

way ANOVA, p values <0.05), although it showed insignificant difference with plasmid E at day 8 post-transfection (Student t test, p values 0.225)

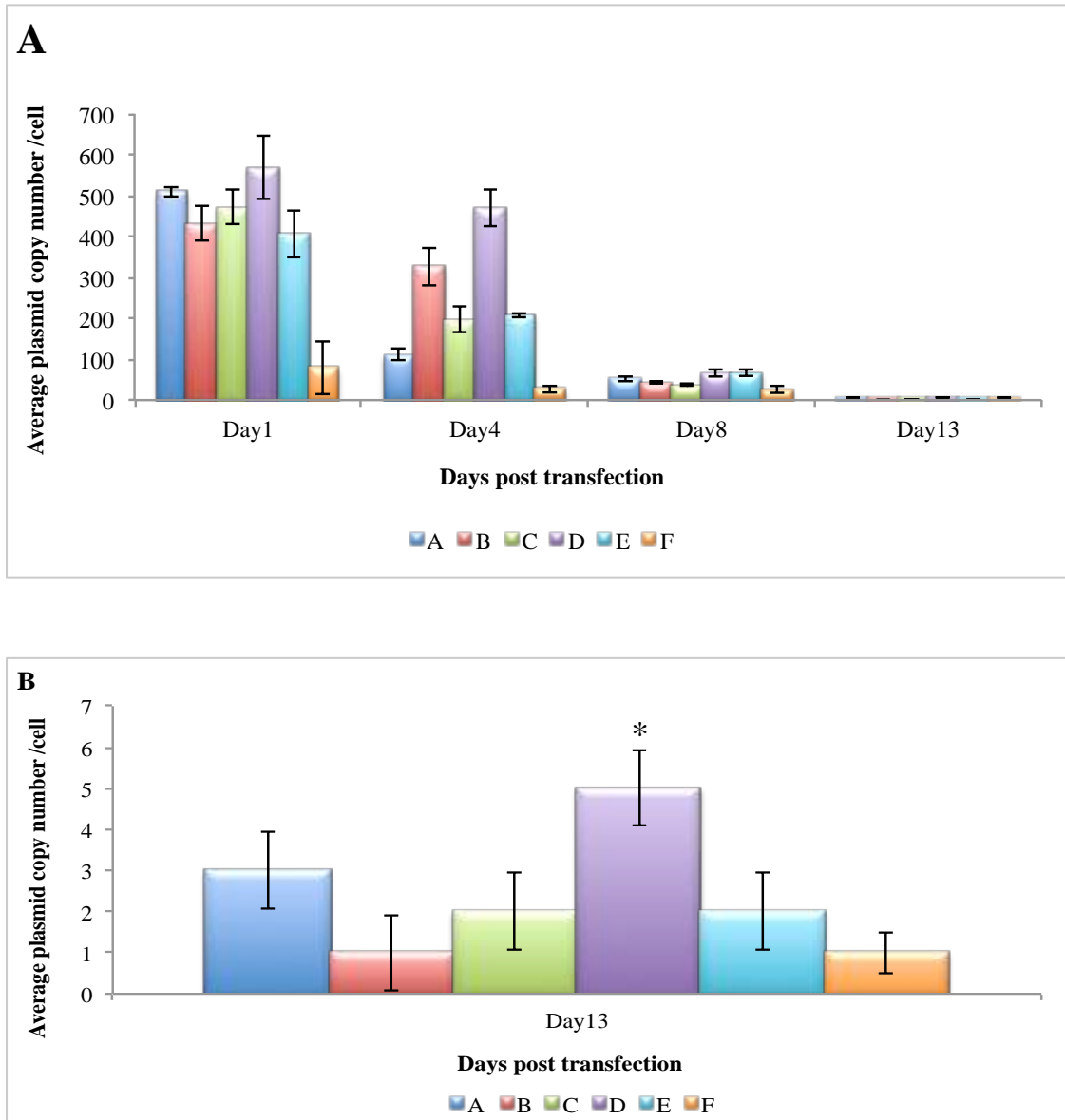
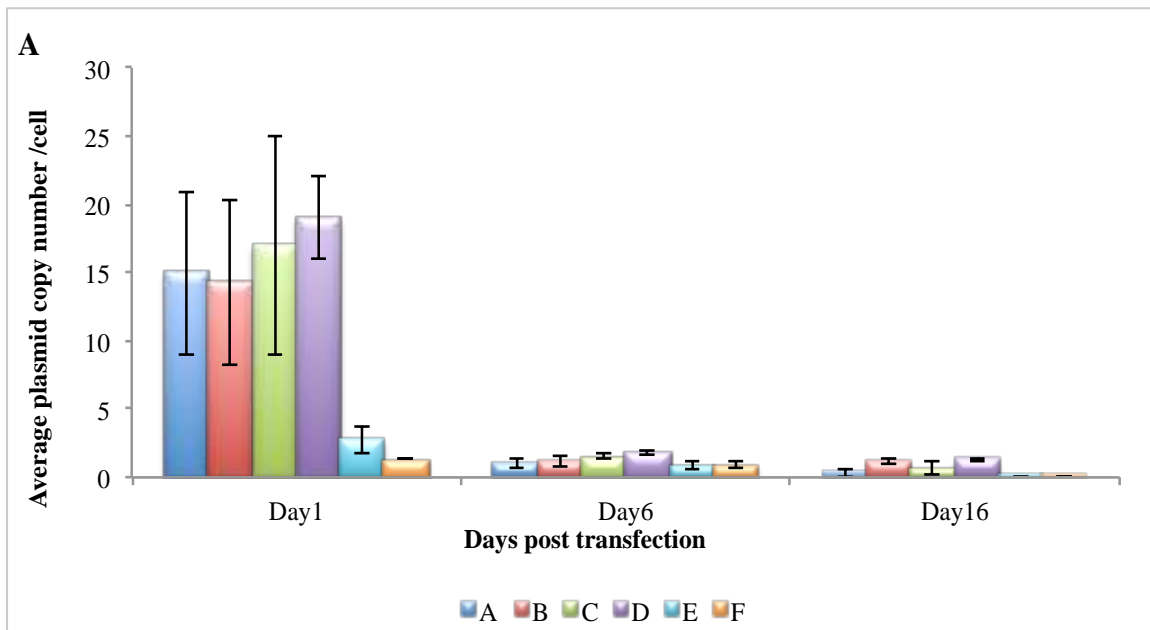


Figure 15: Plasmid stability in H1299 cells. A) Average plasmid copy number of test plasmids (A, B, C, D and E) and the negative control plasmid (F) ($n=3$) at 1, 4, 8 and 13 days post-transfection. B) Average plasmid copy number at 13 days post-transfection. Plasmid D obtained the highest copy number (5 copies per cell) at day 13 post-transfection compared to test and negative control plasmids (one-way ANOVA, p values <0.05). Error bars represent standard deviation (SD).

Plasmid stability in HEK293 cells

Equal amounts of isolated DNA from each collection point (day 1, day 6 and day16) were analyzed by quantitative PCR (qPCR) using specific primers for PUC19 backbone. Actual plasmid copy number per cell was determined from a standard curve of known plasmid concentration. There was insignificant difference in plasmid copy number between test plasmids at day 1 post-transfection and day 16 post-transfection as p values were >0.05 (one-way ANOVA). However, plasmid F showed a significant reduction in copy number at day 1 compared to the others. Despite the fast drop at day 6 post-transfection, plasmid D is significantly the most stable among the others (one-way ANOVA, p values <0.05), however, it showed insignificant results compared to plasmid C copy number at p values 0.147 (Student *t* test) (Figure 16).



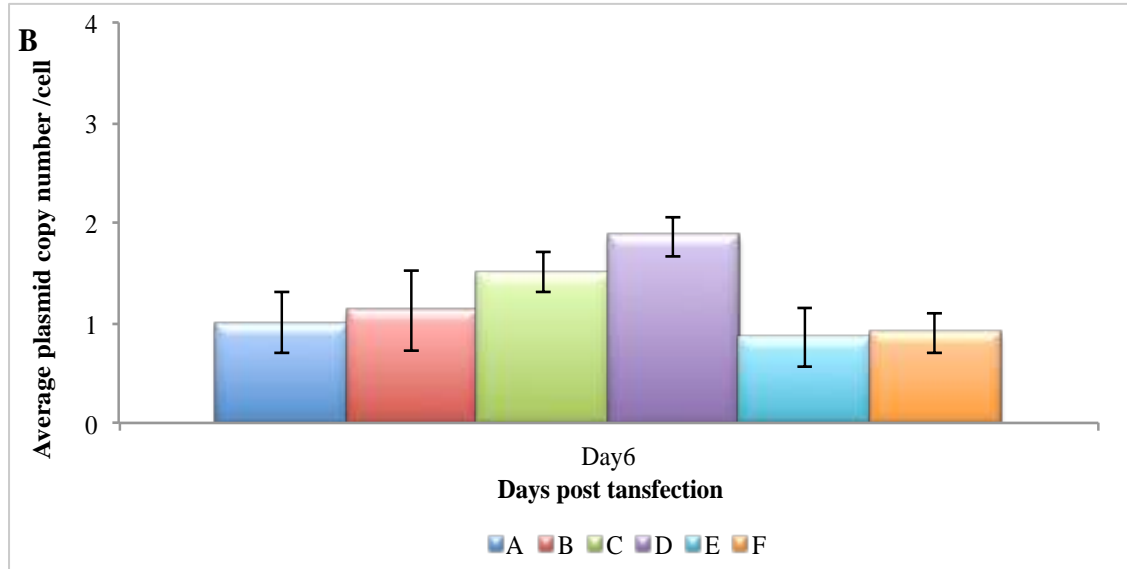


Figure 16: Plasmid stability in HEK293 cells. A) Average plasmid copy number of test plasmids (A, B, C, D and E) and the negative control plasmid (F) (n=3) at 1, 6 and 16 days post-transfection. B) Average plasmid copy number at day 6 post-transfection, plasmid D is significantly the most stable among the others (one-way ANOVA, $p < 0.05$). Error bars represent SD.

Plasmid stability in HeLa cells

QPCR was performed on equal amounts of DNA from each time point (day 6, day 9, day 12 and day 16) to determine the exact plasmid copy numbers in each sample. Absolute copy number of each plasmid per cell was obtained using PUC19 backbone specific primers and a standard curve of known plasmid concentration. From the data, there was no statistically significant change between plasmids copy numbers of the test plasmids and the negative control at all days post-transfection (one-way ANOVA, p values > 0.05) except day 6 post transfection. Although plasmid E was the highest copy numbers at day 12 post-transfection, it is still insignificantly higher than plasmid C and plasmid D (Student t test, p values 0.356 and 0.081, respectively) (Figure 17).

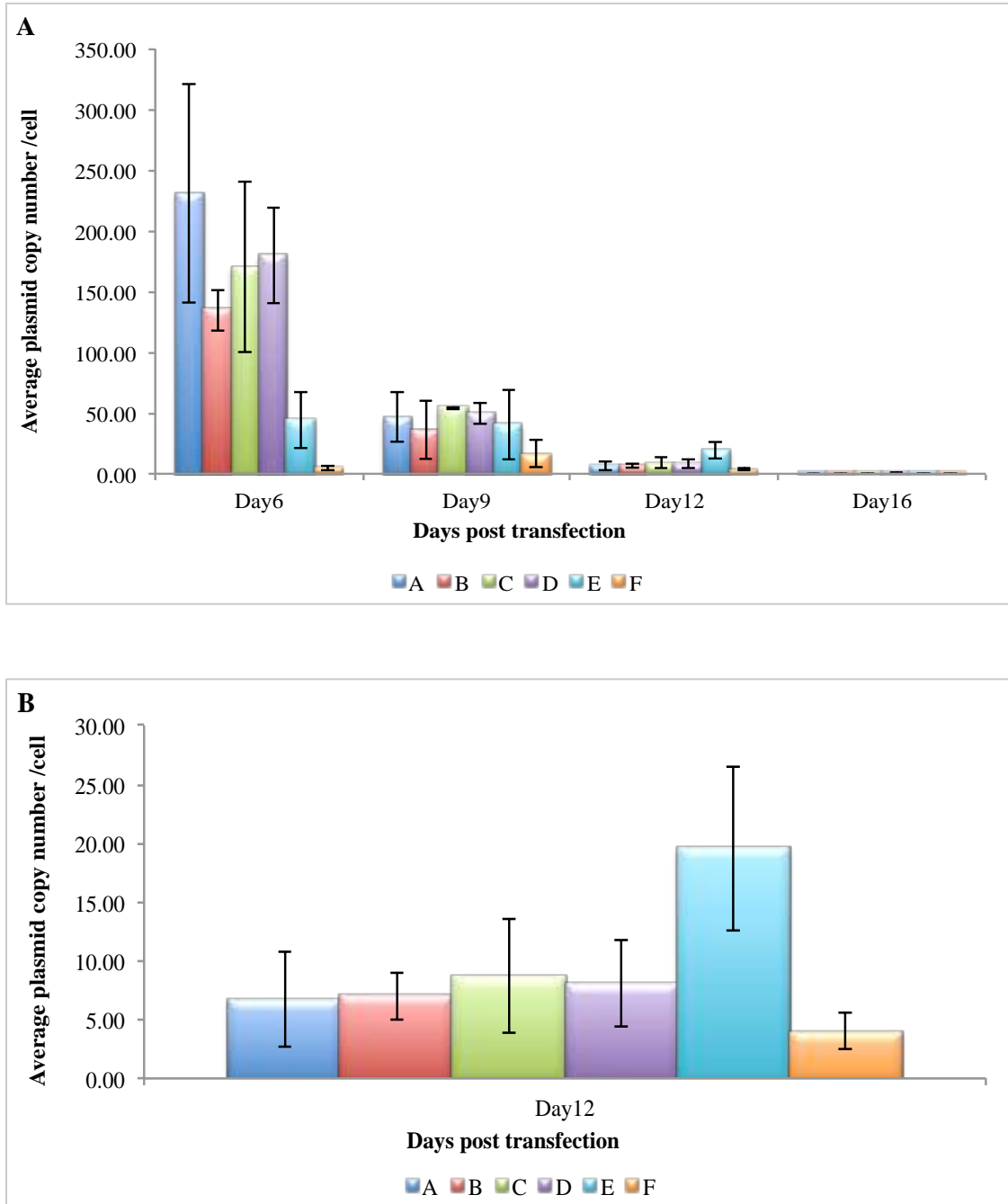


Figure 17: Plasmid stability in HeLa cells. A) Average plasmid copy number per cell of test plasmids (A, B, C, D and E) and the negative control plasmid (F) (n=3) at 6, 9, 12, 16 days post-transfection. B) Average plasmid copy number at day 12 post-transfection, plasmid E is significantly the highest (one-way ANOVA, $p < 0.05$), however it is insignificantly higher than plasmid D (Student t test, p values > 0.05). Error bars represent SD.

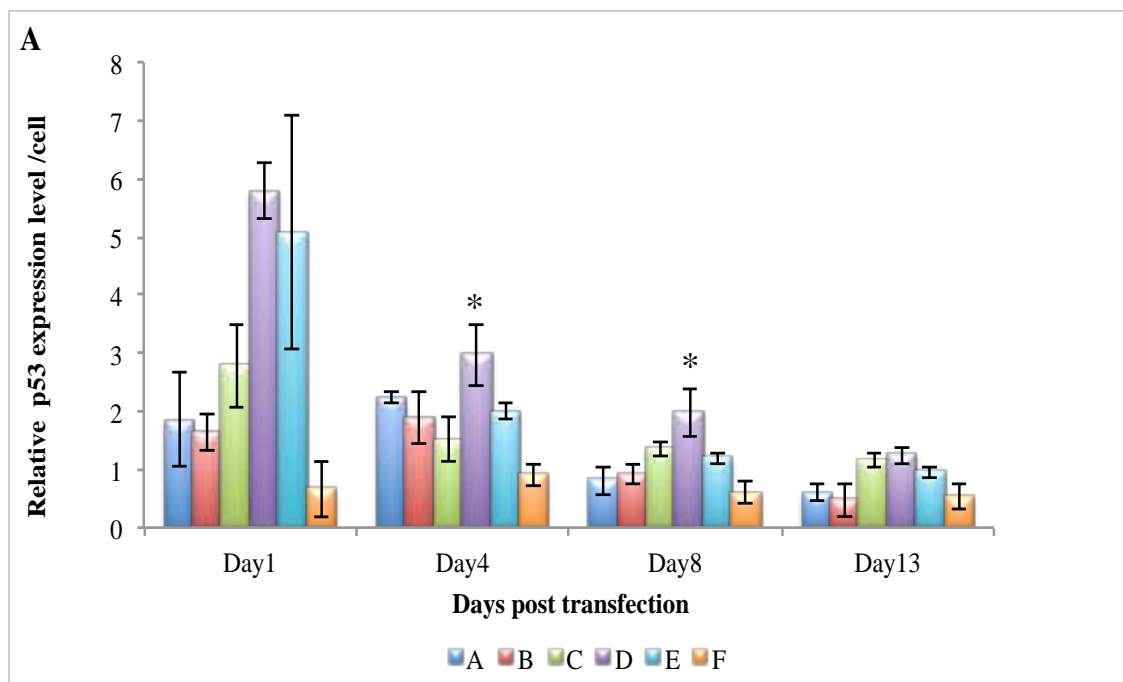
Evaluation of p53 expression level in H1299, HEK293, and HeLa cell lines

To assess p53 transgene expression level and correlate it with pDNA stability, the same plasmids A, B, C, D, E and F as shown in Table 5 were used in this experiment. Since each plasmid has a different molecular size, equal copy numbers from each of the plasmids were transfected into the same three cell lines H1299, HEK293, and HeLa for reliable results. Transfection was done in a 24-well plate with three biological replicates for each plasmid using Lipofectamine 2000 and at 6 hours post-transfection the medium was replaced. Different time course transfections for each cell line were conducted since they differ in their growth rate. The collected samples were utilized to isolate DNA and RNA from a single sample. Then both were quantified and used afterwards for further analysis. The data were analyzed using a one-way ANOVA test as well as Student t test. P values <0.05 considered significant.

P53 expression level in H1299 cells

All isolated and quantified RNAs samples were treated with DNase and cleaned up afterwards. Equal amount of cleaned RNAs were then subjected to reverse transcriptase PCR (RT-PCR) reaction to generate corresponding synthesized first complementary DNAs (cDNAs) that were used as a template for qPCR. Relative p53 mRNA abundance per cell was obtained at each time point (day 1, day 4, day 8 and day13) by the $\Delta\Delta CT$ comparative threshold method using a housekeeping gene that is constantly expressed in the cell for normalization. The levels of p53 were measured based

on the equation $2^{(Ct\ S15 - Ct\ P53)}$. As shown in Figure 18, the p53 expression level that was obtained from plasmid D at day 1 post-transfection was insignificant compared to plasmid E (Student *t* test, *p* values 0.595). The same insignificant difference was observed at 13 post-transfection compared to plasmid D (Student *t* test, *p* values 0.068), although it was significantly higher compared to the others at the same named days (one-way ANOVA, *p* values <0.05). While on 4 and 8 days post-transfection, plasmid D revealed significant superiority over all the other plasmids with the highest transgene expression level among the others (one-way ANOVA, *p* values <0.05).



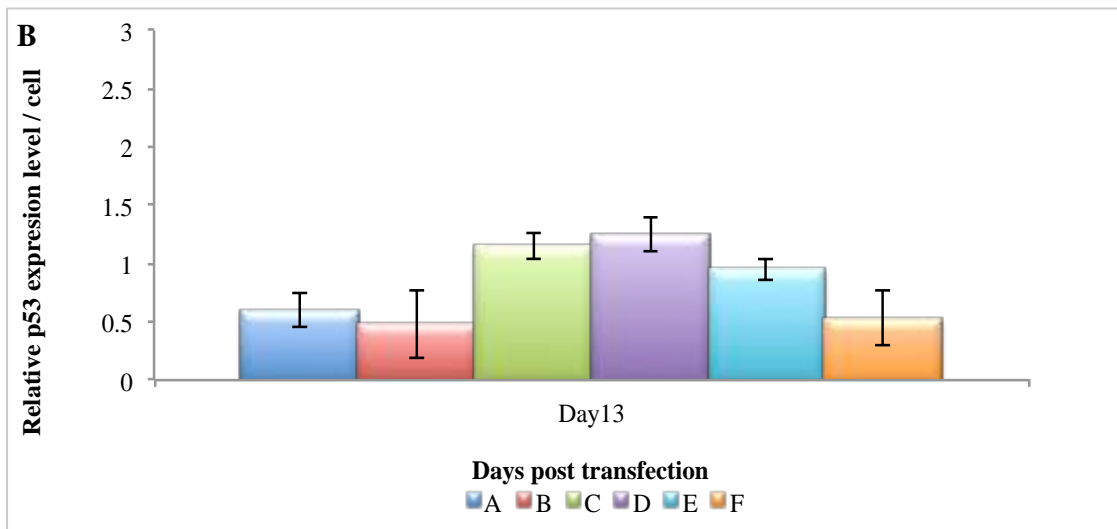


Figure 18: P53 expression in H1299 cells. A) Relative p53 expression level per cell of test plasmids (A, B, C, D and E) (n=3) at 1, 4, 8 and 13 days post-transfection. B) Relative p53 expression level per cell at 13 days post-transfection. P53 mRNA levels were normalized with endogenous control S15 gene and calculated using the equation $2^{-(Ct\ S15 - Ct\ P53)}$. Plasmid D showed significant increase in p53 expression relative to cells transfected with the other test plasmids and negative control plasmid at days 4 and 8 post-transfection (one-way ANOVA, p values <0.05). Error bars represent SD.

P53 expression level in HEK293 cells

The isolated RNAs were treated with DNase for DNA digestion of any residues in the samples. They then underwent DNase clean up to remove the remaining enzyme. Equal amounts of total RNA from each sample were used as a template for reverse transcriptase PCR reaction. Then, qPCR was performed on equal amounts of cDNA using p53 specific primers. The relative measurement of p53 mRNA level was obtained at each time point (day 1, day 6 and day 16) by the $\Delta\Delta Ct$ comparative threshold method using a housekeeping gene for normalization.

As HEK293 cells already express cellular p53, we subtracted the expressed p53 that was obtained from each test plasmid from the expressed p53 that was obtained from the negative control sample to achieve the actual level of exogenous p53 expression. This subtraction was done after they were both normalized with the housekeeping gene. P53 expression that was obtained from the negative control represents the p53 cellular basal level. The equation used was $2^{-(\text{Ct S15}_{\text{test sample}} - \text{Ct P53}_{\text{test sample}} - (\text{Ct S15}_{\text{negative control}} - \text{Ct P53}_{\text{negative control}}))}$, where Ct S15 is the PCR threshold cycle of S15 amplified by S15 specific primers and Ct P53 is the PCR threshold cycle of p53 amplification using specific p53 primers from the same sample.

From the generated data, no significant change was noted in p53 mRNA level of plasmids tested at days 1 and 16 post-transfection, however; plasmid E was the lowest among them. Although the p53 expression level displayed a significant decrease at day 6 post-transfection, plasmid D is still higher than plasmid C (Student *t* test, *p* values <0.05). Also, it is significantly the highest compared to the others (one-way ANOVA, *p* values <0.05) (Figure 19).

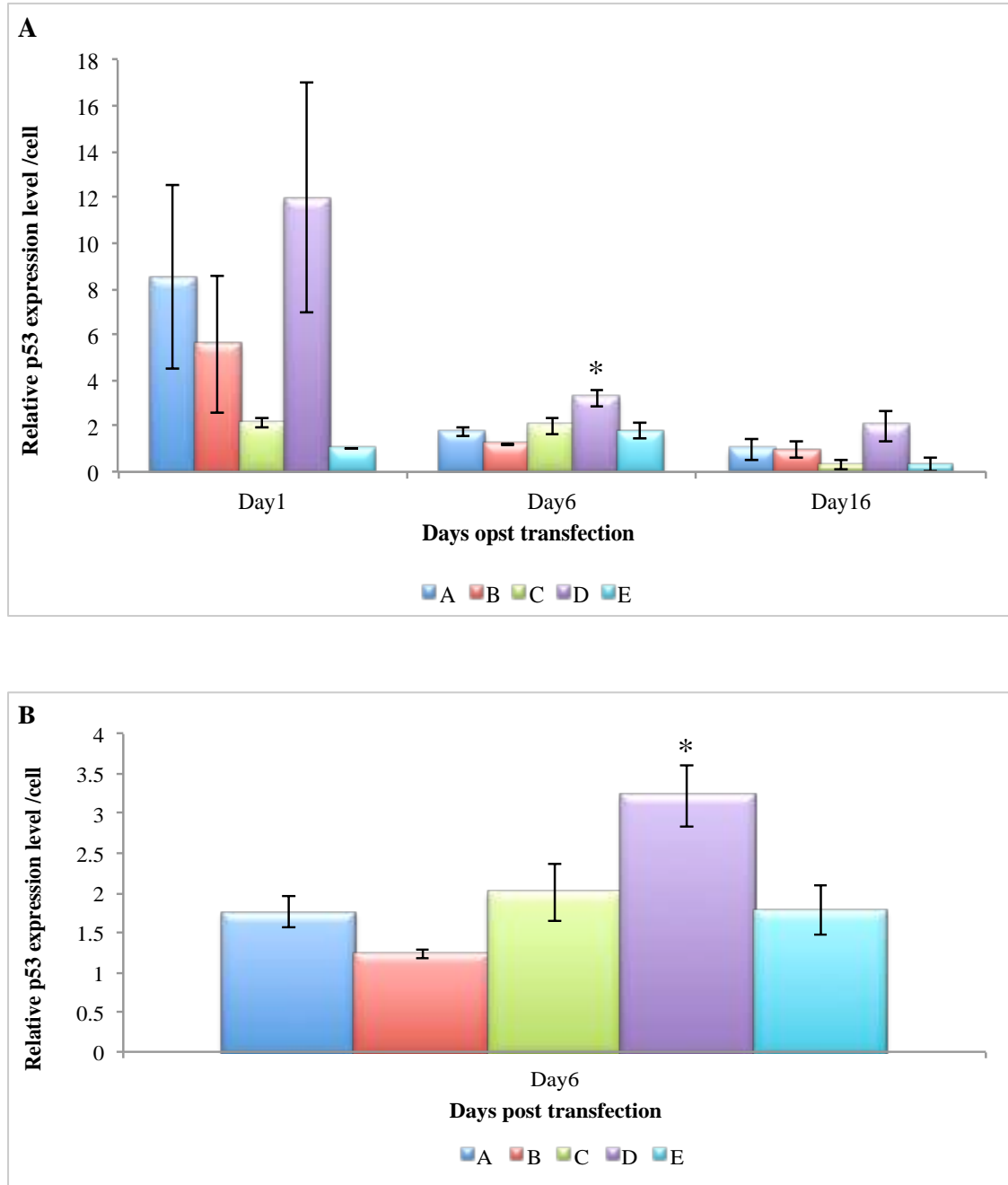


Figure 19: P53 expression in HEK293 cells. A) Relative p53 expression level per cell of test plasmids (A, B, C, D and E) after normalization with p53 cellular background at 1, 6 and 16 days post-transfection (n=3). B) Relative p53 expression level per cell at 6 days post-transfection. P53 mRNA levels were normalized with endogenous control S15 gene and calculated using the equation $2^{-(Ct\ S15 - Ct\ P53) - (Ct\ S15 - Ct\ P53)}$. At day 6, Plasmid D is significantly the highest among the others (one-way ANOVA, p values <0.05). Error bars represent SD.

P53 expression level in HeLa cells

All RNAs that were isolated underwent DNase treatment and clean up afterwards to achieve DNase-free RNAs samples. Equal amount from each sample was then used in a reverse transcriptase PCR reaction to obtain cDNAs that were to serve as a template for qPCR. Actual p53 mRNA level was measured at each time point (day 6, day 9, day 12 and day16) by $\Delta\Delta CT$ comparative threshold method using a housekeeping gene for normalization. Since HeLa cells express p53 at a specific basal level, the level of exogenous p53 mRNA was calculated by subtracting the expressed p53 that was obtained from each test plasmid after normalization with a housekeeping gene from the expressed p53 that obtained from negative control sample after normalization with housekeeping gene as well. The used equation is $2^{-(Ct\ S15_{test\ sample} - Ct\ P53_{test\ sample}) - (Ct\ S15_{negative\ control} - Ct\ P53_{negative\ control})}$, where Ct S15 is the PCR threshold cycle of S15 amplified by S15 specific primers and Ct P53 is the PCR threshold cycle of p53 amplification using specific p53 primers from the same sample.

From the analyzed results, it was found that no significant change was observed between the plasmids at days 6 and 16 post-transfection (one-way ANOVA, p values >0.05). In contrast, at day 9 post-transfection, plasmid D has shown to obtain the highest expression among the plasmids reaching below the significant level, which is 0.05. Also, at day 12 post-transfection, plasmid D is still revealed to be superior over all of the other plasmids (one-way ANOVA, p values <0.05), whereas compared to plasmid E it showed insignificant change at p values 0.145 (Student *t* test) (Figure 20).

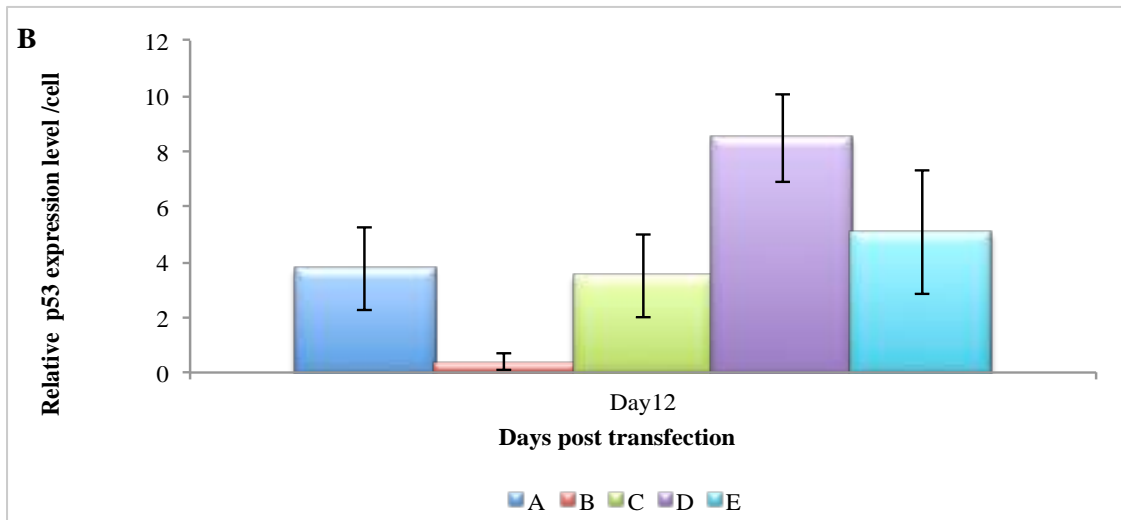
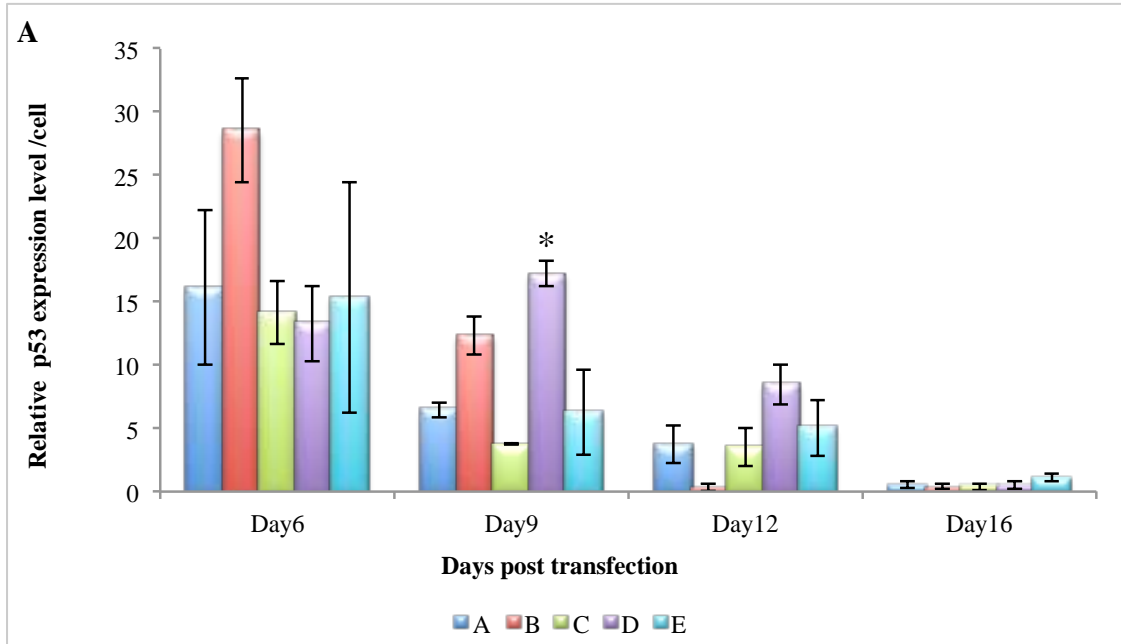


Figure 20: P53 expression in HeLa cells. A) Relative p53 expression level per cell of test plasmids (A, B, C, D and E) after normalization with p53 cellular background at 6, 9, 12, 16 days post-transfection (n=3). A) Relative p53 expression level per cell at 6 days post-transfection. P53 mRNA levels were normalized with endogenous control S15 gene and calculated using the equation $2^{-(Ct\ S15 - Ct\ P53) - (Ct\ S15 - Ct\ P53)}$. At day 12 post-transfection, plasmid D is still revealed to be superior over all of the other plasmids (one-way ANOVA, p values <0.05) with less significance compared to plasmid E. Error bars represent SD.

EVALUATING REPLICATION EFFICIENCY OF Ad5dlE1b55kDa MUTANT IN CELL LINES EXPRESSING VARIOUS LEVELS OF P53

In order to evaluate the replication of Ad5dlE1b55kDa in various cell lines expressing either endogenous or exogenous wild type p53 at various levels, plasmids that expressed different p53 levels (described in chapter I) were used to obtain different p53 backgrounds. To facilitate this experiment, three different viruses were used as shown in Table 9: (i) Ad5dlE1b55kDa (Ad5dlE1b), an Ad with a deletion of E1B55kDa region (827bp) and E3 region, that lacks the ability to interact with p53 but still contains E1B 19kDa to prevent premature death of host cell through apoptosis and to achieve transformation, (ii) E1 deleted Ad (AdGFP), an Ad with a deletion of E1 and E3 regions and encoding the GFP reporter gene, that lacks the ability of replication, and (iii) wild type Ad (Ad5wt), an Ad with a deletion of E3 region only. Since the E3 region expresses the death protein, it was deleted in all viruses to avoid the early death of the infected cell and to facilitate viral replication. Both AdGFP and Ad5wt vectors were used as controls, in addition to PUC19 transfected cells that were used as negative controls in the experiment.

In this chapter, two distinct cell lines with and without p53 expression: HeLa (wt-p53) and H1299 (null-p53) were used. Before evaluating the effect of different p53 levels on viral replication, we tried to assess viral growth in the two cell lines. Therefore, the replication kinetics was characterized for each virus listed in Table 9 in both cell lines through viral infection of (Ad5dlE1b, AdGFP and Ad5wt) at MOI of 10 PFU/cell, which

was optimized in another experiment for the highest number of viral particles that didn't cause cell detachment from the monolayer (Data not shown). Viral DNA synthesis was used as an indicator of replication efficiency.

Then, the viral replication efficiency in cells expressing various levels of p53 was determined. An *in vitro* transfection of different molar ratio from each plasmid (A, B, C, D, E and F) to establish different p53 levels in both cell lines was conducted in a 24-well plate using Lipofectamine 2000 reagent and medium was replaced six hours post-transfection. Following transfection, viral infection of adenoviruses (Ad5dlE1b, AdGFP and Ad5wt) was performed at 24 hours post-transfection. Triplicate samples were collected at different time points 2, 24, 48 and 72 hours post-infection and total DNA and RNA were isolated from the same sample to assess viral DNA replication efficiency as well as p53 expression level. The data were analyzed using the one-way ANOVA test as well as the Student t test.

Table 9: List of adenoviruses and p53 plasmids used in this chapter

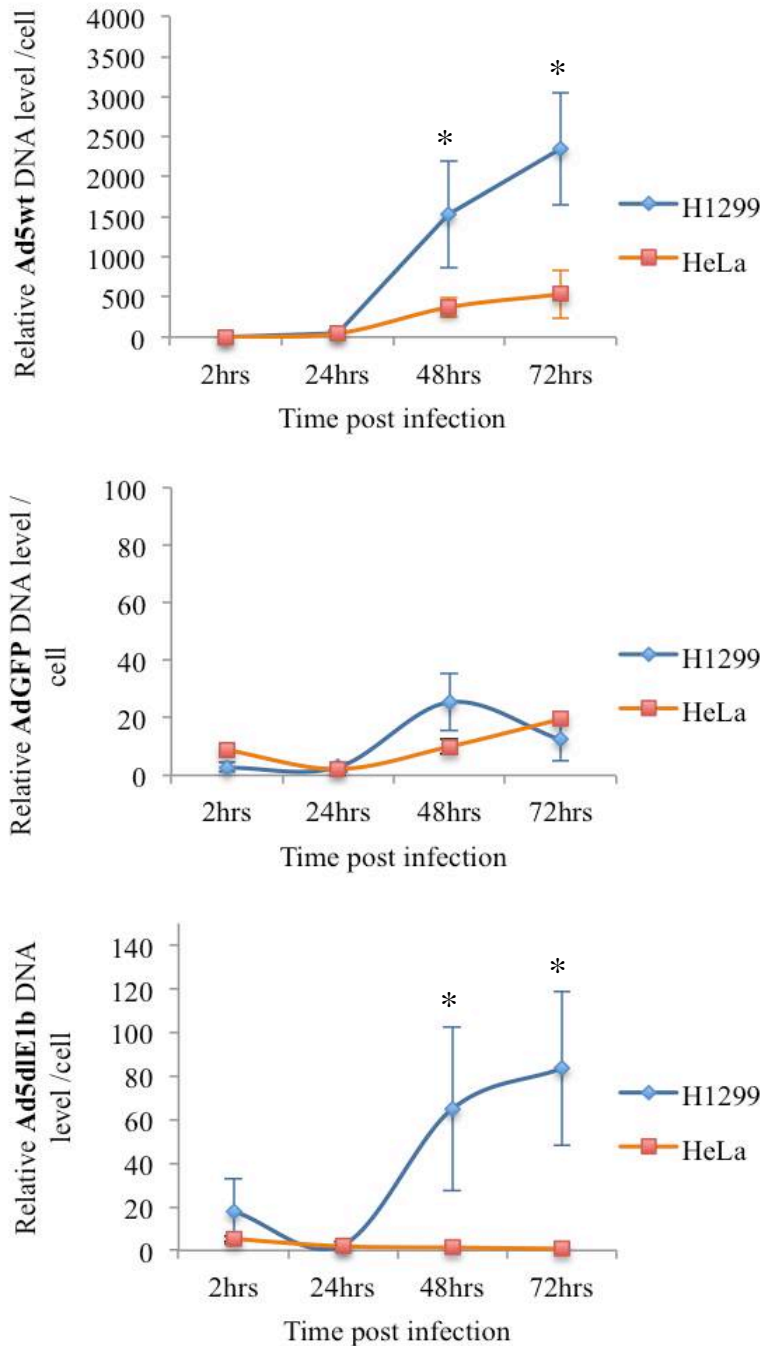
Virus	Contents	Source	Reference
Ad5dlE1b	dl E1b 55kDa + dl E3	Dr. Haj-Ahmad	(Haj-Ahmad, 1986)
AdGFP	dl E1 + dlE3 + GFP gene	Dr. Misic	(Misic, 2013)
Ad5wt	dl E3	Dr. Haj-Ahmad	(Haj-Ahmad, 1986)
Plasmid abbreviation	Plasmid name	Contents	Reference
A	PA	P53 gene	Figure 12
B	PB	P53 gene +MARI	Figure 13
C	PC	P53 gene +MARI+MARII	Figure 13
D	PD	P53 gene +MARI+MARII+HPRE	Figure 14
E	PE	P53 gene +MARI+HPRE+MARII+Poly-A	Figure 14
F	PF	Negative control	-

Characterization of Ad5wt, AdGFP and Ad5dlE1b55kDa replication in H1299 and HeLa cells

To compare viral replication kinetics of Ad5wt, AdGFP and Ad5dlE1b55kDa in relation to cell types, the replication phenotype of each virus was characterized in both H1299 and HeLa cell lines. Replication efficiency was determined by measuring relative DNA levels of all viruses by qPCR, which was performed on the same amounts of DNA samples using adenovirus probe and primers specific for the hexon gene. Delta C_T (ΔC_T) threshold method was applied afterwards on the raw PCR C_T using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 5S as housekeeping genes for normalization to calculate the viral DNA level per cell. The measurements were assessed using the equation $2^{(C_T \text{ cellular gene} - C_T \text{ hexon})}$. Moreover, viral cytopathic effect (CPE) of different adenoviruses was determined morphologically using the inverted microscope.

The results show that growth rate of Ad5wt and Ad5dlE1b55kDa was significantly higher in H1299 that is null for p53 expression than HeLa cells, which contains wt p53 expression at all time post-infection (Student t test, p value >0.05). Also, the same result was found in the case of AdGFP at 48 hrs post-infection, however, a reverse was true at 2 hrs post-infection. This was demonstrated through the calculation of viral relative growth percentage in H1299 to HeLa cells. Also it was observed in the cellular morphology of both cells using the microscope, as CPE causes changes in the structure of the infected cells reaching cell detachment depending on the efficiency of viral production (Figure 22). These results showed the effect of p53 on viral replication efficiency. The viral DNA replication was greatest at 72 hours post-infection (Figure 21).

A



B

Ad5wt	%
2 hours	53
24 hours	119
48 hours	412
72 hours	439

AdGFP	%
2 hours	32.2
24 hours	132
48 hours	254
72 hours	63

Ad5dlE1b	%
2 hours	345
24 hours	128
48 hours	4907
72 hours	10431

Figure 21: Growth rate of Ad5wt, AdGFP and Ad5dlE1b in H1299 and HeLa cells. A) Relative viral DNA levels in both H1299 and HeLa cellular backgrounds over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with cellular gene using the equation $2^{(Ct \text{ cellular gene} - Ct \text{ hexon})}$. Error bars represent SD. B) Ad5wt, AdGFP and Ad5dlE1b relative growth percentage in H1299 to HeLa cells.

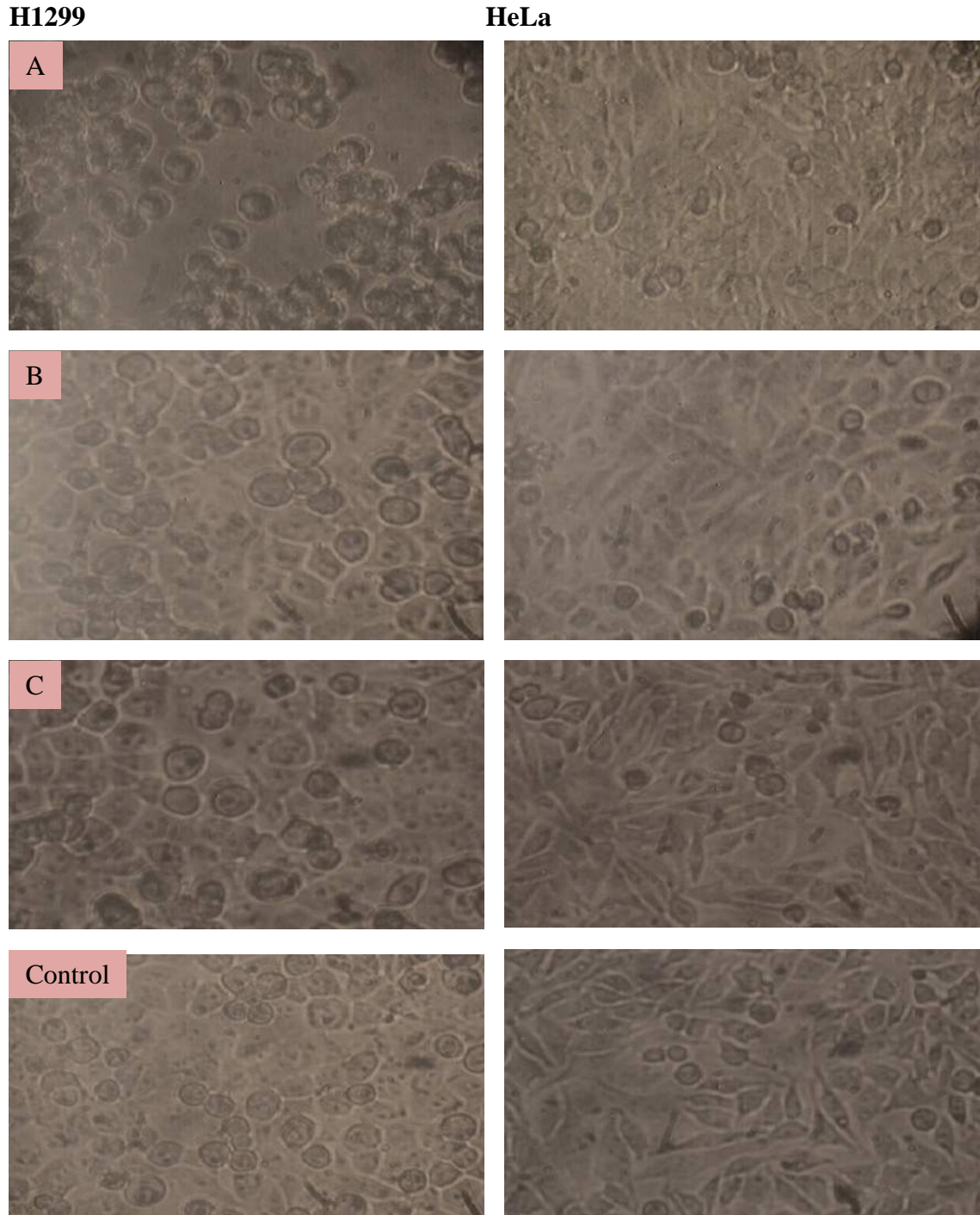


Figure 22: Cellular morphology of infected H1299 and HeLa cells with Ad5wt, AdGFP and Ad5dlE1b55kDa at 72 hours post-infection. A) cells infected with Ad5wt at MOI of 10 PFU/cell, B) AdGFP infection of both H1299 and HeLa at MOI of 10 PFU/cell, C) Ad5dlE1b55kDa infected cells at MOI of 10 PFU/cell, and the control (non-infected).

Effect of p53 levels on Ad5wt, AdGFP and Ad5dlE1b55kDa replication efficiency in H1299 cells

As mentioned above, in this part, H1299 cell line was first transfected with multiple p53 expression vectors to obtain various levels of p53. Second, viral infection using the three viruses was carried out at 24 hours post-transfection. Viral DNA replication efficiency of all viruses was determined using isolated DNA of the different samples. In addition, isolated RNA from the same sample was converted to cDNA through the use of RT reaction and then used in determining p53 expression level. Probe based-TaqMan qPCR was performed on equal amounts of both DNA and cDNA using specific probe and primers for adenovirus hexon gene and p53gene, respectively. Relative viral DNA levels were assessed by Δ CT method through normalization with cellular GAPDH gene at different collection points, whereas relative p53 expression per cell was obtained using the same Δ CT method but with the S15 housekeeping gene for normalization. The level of viral DNA and p53 mRNA were measured according to the equations $2^{-(Ct\text{ GAPDH} - Ct\text{ hexon})}$ and $2^{-(Ct\text{ S15} - Ct\text{ P53})}$, respectively.

Determination of Ad5wt replication efficiency

From the plotted data in Figure 23, a relative relationship between the expression of wild type p53 that obtained from different plasmids (A-E) and Ad5wt DNA replication was observed. However, variant amount of p53 didnot affect the ability of viral replication. Viral DNA reached the highest level in H1299 cells that lacked p53 expression (F). However, the increase was insignificant compared to viral DNA levels in H1299 cells with different p53 expression backgrounds from plasmids (A-E) at p values

0.837 (one-way ANOVA). The highest obtained peaks of p53 expression differ between the plasmids as some were at 2 hours whereas the others were at 24 and 48 hours post-infection, which is not the case of negative control (F).

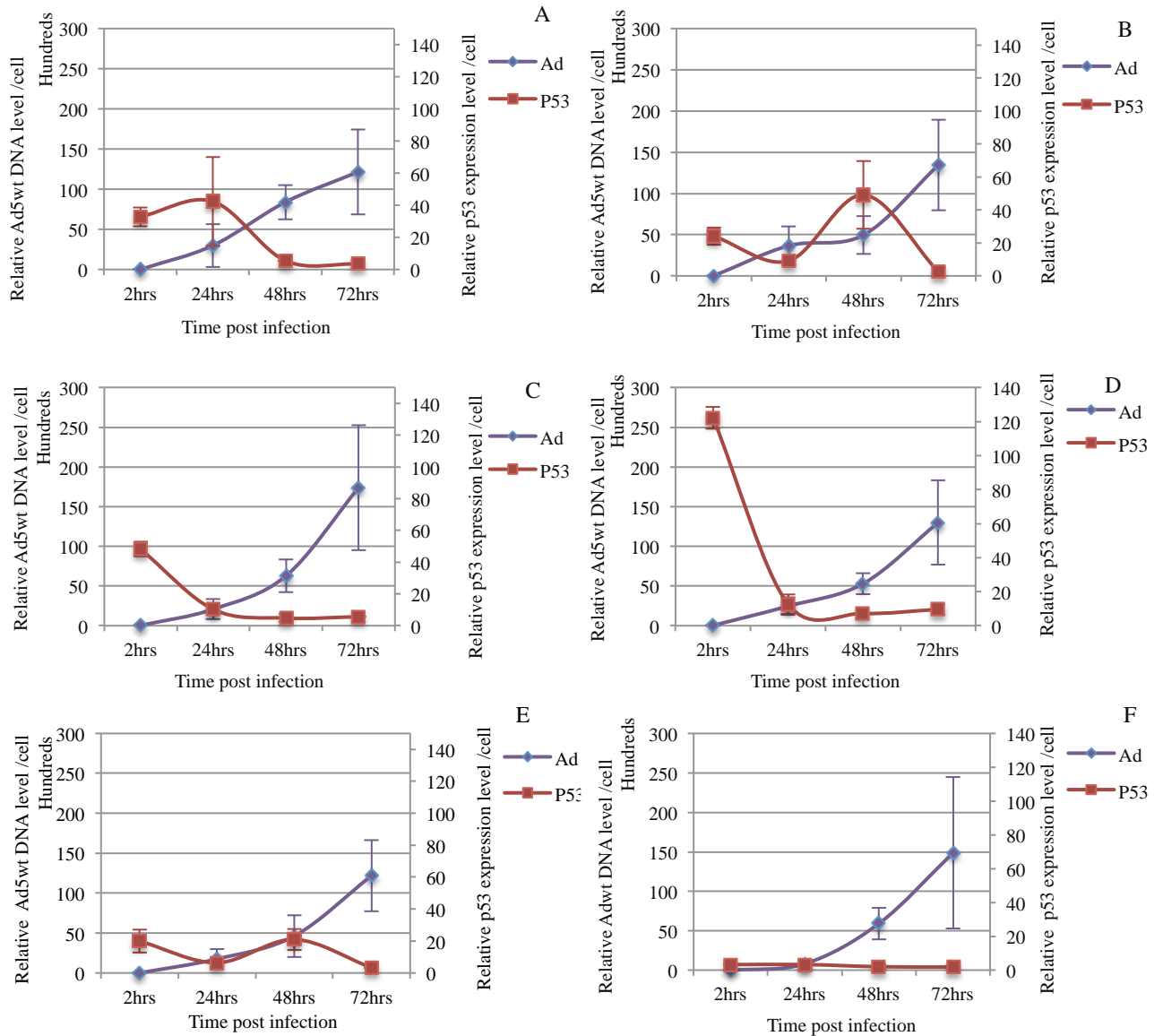


Figure 23: Effect of p53 levels on Ad5wt replication efficiency in H1299 cells. Relative Ad5wt DNA levels in different p53 expression levels that obtained from plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with 5S using the equation $2^{(Ct\ GAPDH - Ct\ hexon)}$. Relative p53 expression levels were normalized using the expression of S15 gene and measured using the equation $2^{(Ct\ S15 - Ct\ P53)}$. Error bars represent SD.

Determination of AdGFP replication efficiency

As seen in Figure 24, there was a weak correlation between AdGFP replication and wild type p53 expression level that expressed from different plasmids (A-E) in H1299 cells. Viral DNA level reached the highest under p53 level that expressed from plasmid B. However, this increase in the replication capacity was insignificant compared to viral replication in the other different p53 backgrounds that obtained from plasmids (A-F), as the p values was >0.05 (one-way ANOVA) at 72 hours post-infection. On the other hand, there was a significant difference in DNA levels of AdGFP compared to Ad5wt. P53 expression was shown to reach its maximum at 2 and 24 hours post-infection in all cases (A-E). Although plasmid (F) does not encode a p53 transgene, a very little amount of p53 expression was observed at 24 hours post-infection for unknown reason.

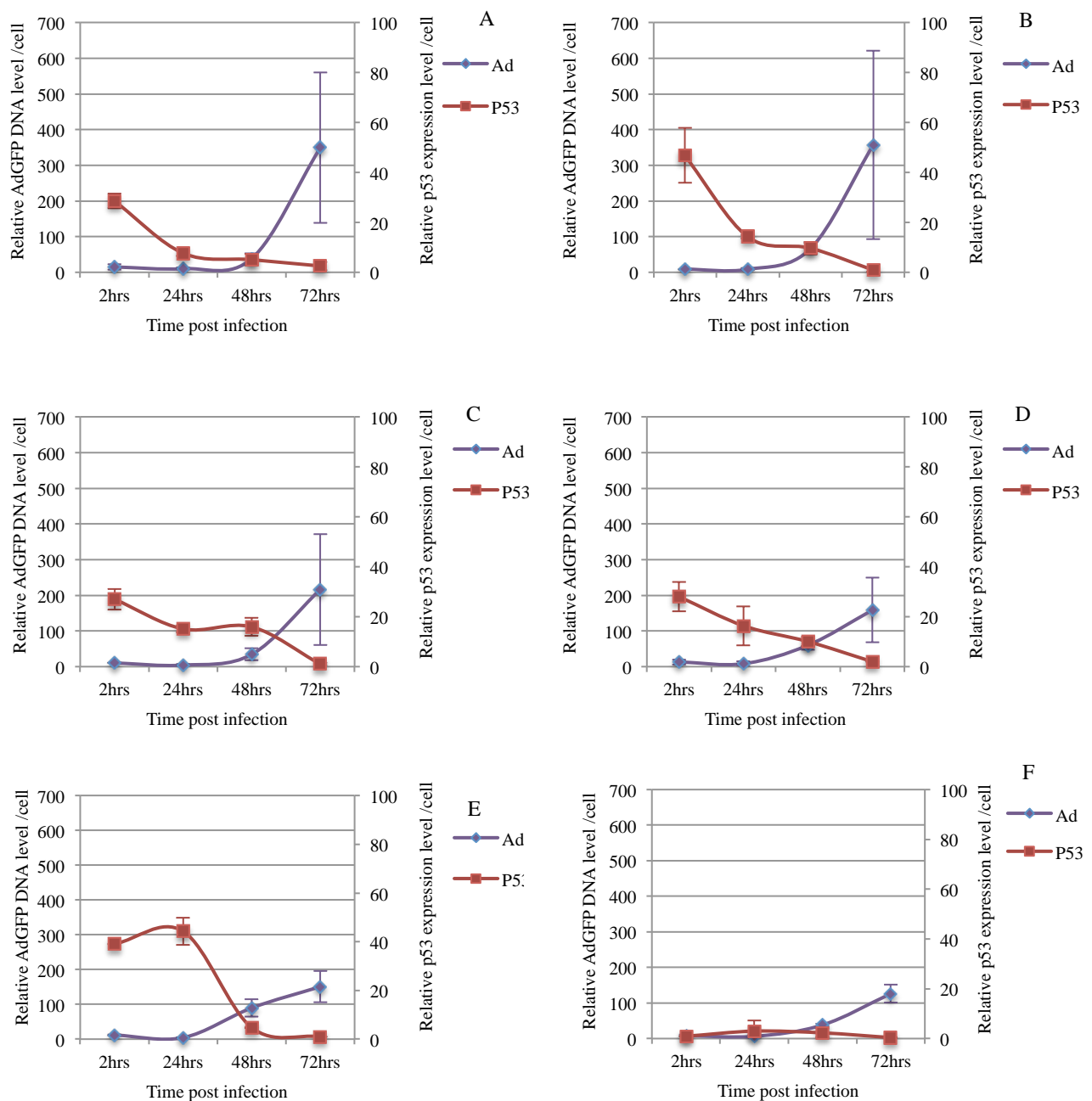


Figure 24: Effect of p53 levels on AdGFP replication efficiency in H1299 cells. Relative AdGFP DNA levels in different p53 expression levels that obtained from plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with 5S using the equation $2^{(Ct \text{ GAPDH} - Ct \text{ hexon})}$. Relative p53 expression levels were normalized using the expression of S15 gene and measured using the equation $2^{(Ct \text{ S15} - Ct \text{ P53})}$. Error bars represent SD.

Determination of Ad5dlE1b55kDa replication efficiency

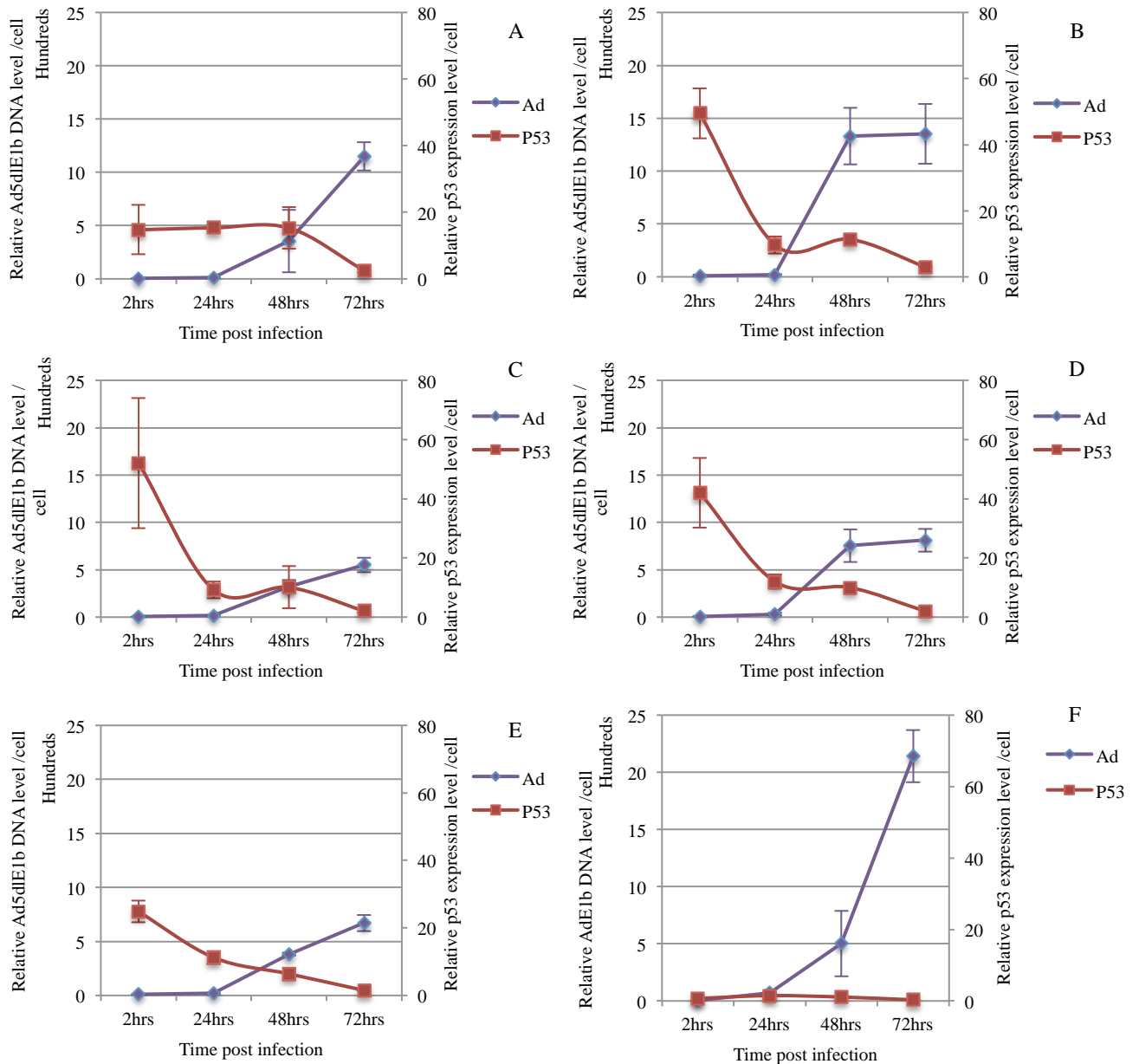


Figure 25: Effect of p53 levels on Ad5dlE1b replication efficiency in H1299 cells. Relative Ad5dlE1b DNA levels in different p53 expression levels that obtained from plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with 5S using the equation $2^{(Ct \text{ GAPDH} - Ct \text{ hexon})}$. Relative p53 expression levels were normalized using the expression of S15 gene and measured using the equation $2^{(Ct \text{ S15} - Ct \text{ P53})}$. Error bars represent SD.

The trend of Ad5dlE1b55kDa replication was in correlation with the trend of p53 expression in all p53 backgrounds that obtained from different plasmids (A-E). In H1299 transfected with negative control (F), viral DNA level increased to the highest in the absence of p53 expression. In the same case, it started to increase earlier at 24 hours post-infection whereas it increased significantly at 48 hours post-infection in all cases, but Ad5dlE1b55kDa DNA level in the case of plasmid B was significantly higher than this window (A, C, D, E and F) at p value <0.05 (one way ANOVA). However, at 72 hours post-infection, there was insignificant difference in viral DNA levels under different p53 expression levels from plasmids (A-E) (one-way ANOVA, p value was 0.194) whereas when compared to the negative control, the viral DNA level reached the highest among the others with a p values <0.05 (one-way ANOVA). Generally, Ad5dlE1b55kDa DNA level was significantly lower than Ad5wt and actually significantly higher than AdGFP. P53 expression level reached its peak at 2 hours post-infections in all cases and then started to decrease until it reached zero at 72 hours post-infection. This observation was not applied to the negative control (F).

Effect of p53 levels on Ad5wt, AdGFP and Ad5dlE1b55kDa replication efficiency in HeLa cells

To examine if the same trend of viral replication was observed in HeLa cells, relative DNA levels of all viruses were determined. QPCR was performed on the DNA samples using adenovirus probe and primers against the hexon gene. ΔC_T threshold method was utilized afterwards on the raw PCR C_T using the 5S gene for normalization to calculate the viral DNA level. The measurements were done through the equation $2^{(Ct\ 5S - Ct\ hexon)}$. On the other hand, relative p53 expression level was also determined using previously cleaned RNAs from the same sample. cDNA was generated through the use of RT reaction and then subjected to qPCR using specific p53 probe and primers. The level of expressed p53 per cell was measured by applying ΔC_T threshold method using the equation $2^{(Ct\ S15 - Ct\ P53)}$.

Determination of Ad5wt replication efficiency

From Figure 26, a relative relationship was observed under all p53 backgrounds that obtained from endogenous and exogenous expression. Viral DNA level started to increase at different time points and thus reached various levels at 72 hours post-infection. However, insignificant difference of viral DNA levels was obtained from cells expressing endogenous and exogenous p53 (plasmids A-E) and cells endogenously expressing p53 (F, negative control) at 48 hours post-infection (one-way ANOVA, p values >0.05). The p53 expression reached its peak at 2 and 24 hours post-infection and then started to decrease until it reached the cellular basal.

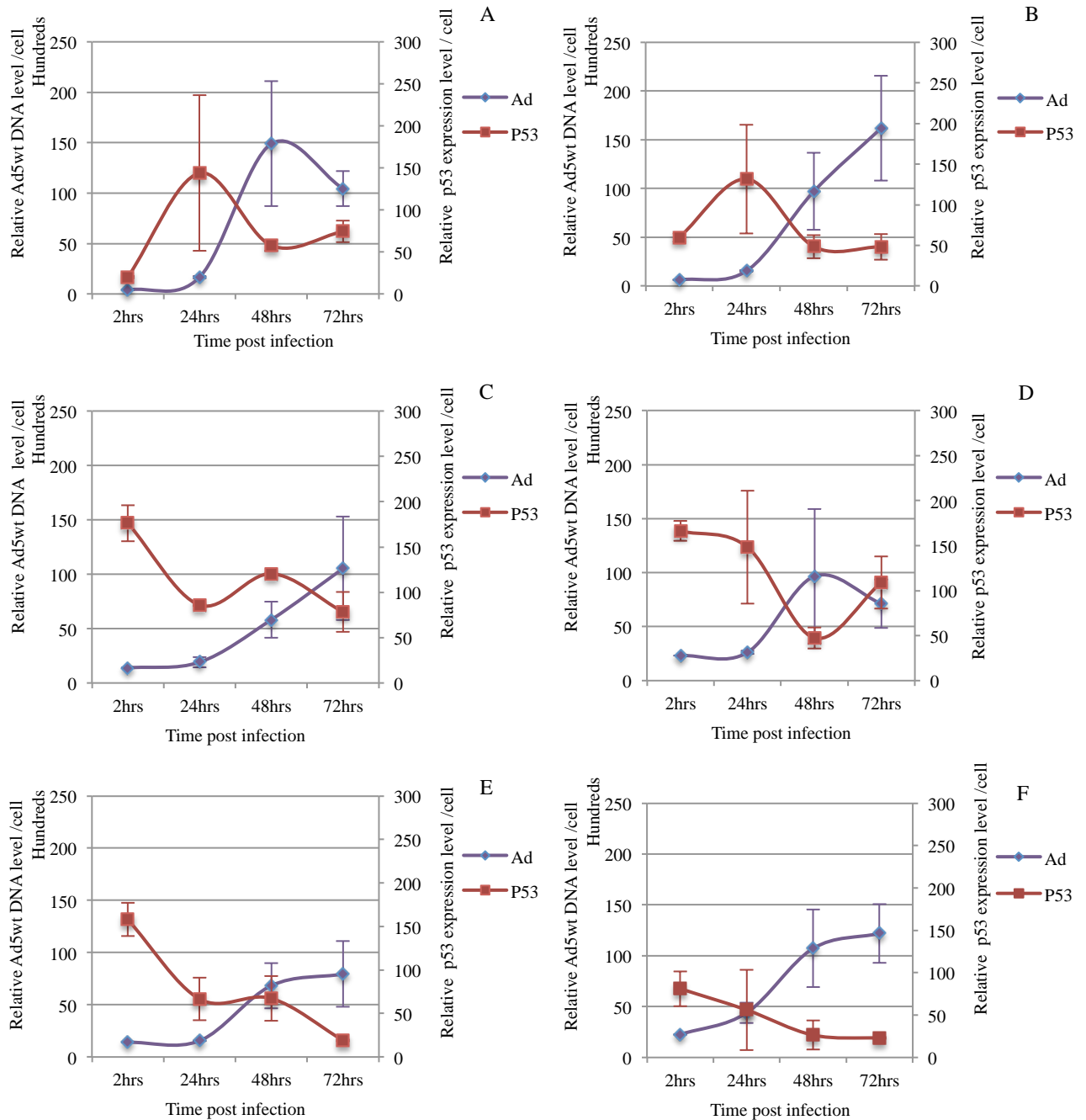


Figure 26: Effect of p53 levels on Ad5wt replication efficiency in HeLa cells. Relative Ad5wt DNA levels in different p53 expression levels that obtained from HeLa cells transfected with plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with 5S using the equation $2^{(Ct\ 5S - Ct\ hexon)}$. Relative p53 expression levels were normalized using the expression of the S15 gene and measured using the equation $2^{(Ct\ S15 - Ct\ P53)}$. Error bars represent SD.

Determination of AdGFP replication efficiency

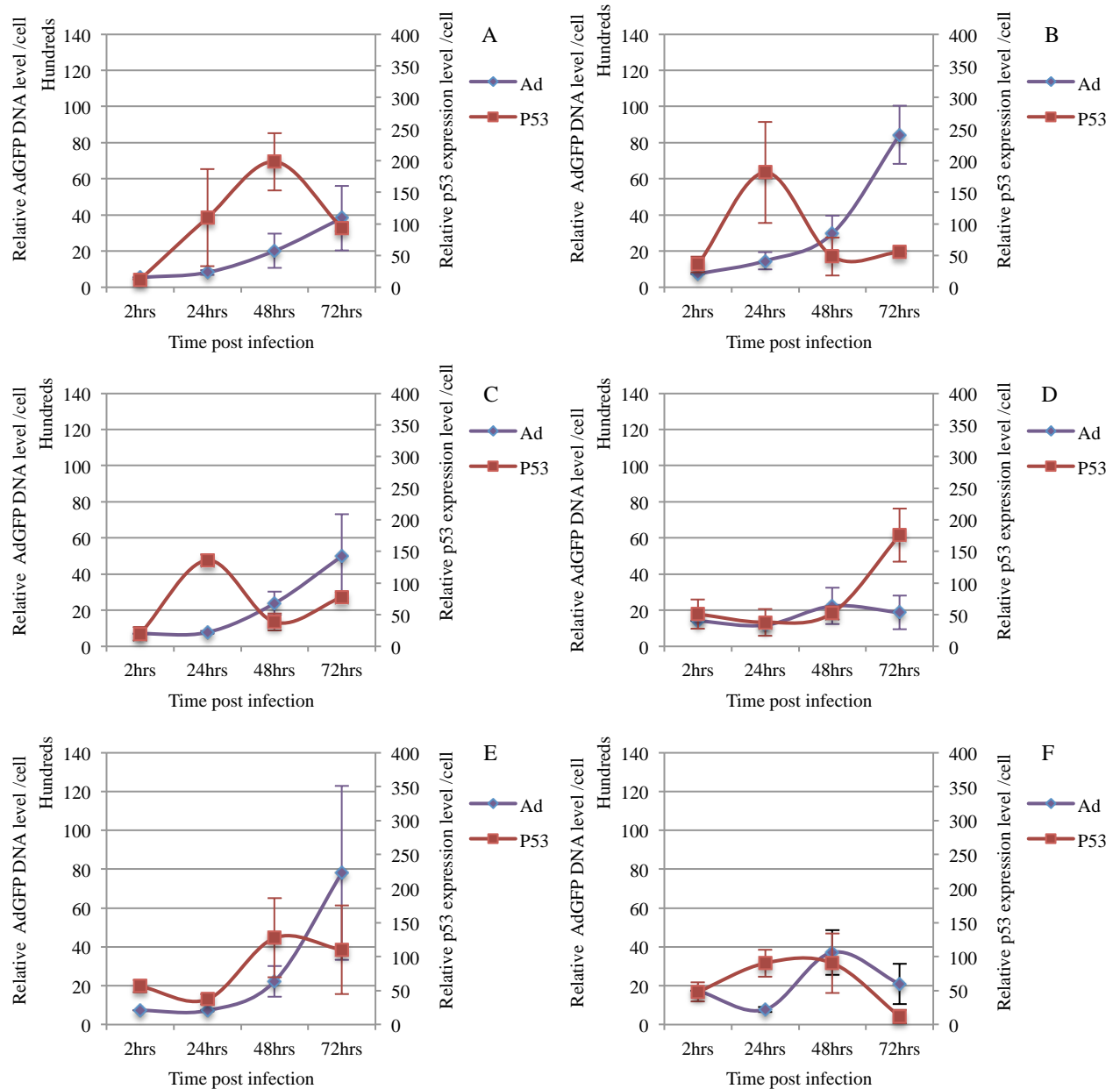


Figure 27: Effect of p53 levels on AdGFP replication efficiency in HeLa cells. Relative AdGFP DNA levels in different p53 expression levels that obtained from HeLa cells transfected with plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with 5S using the equation $2^{(Ct\ 5S - Ct\ hexon)}$. Relative p53 expression levels were normalized using the expression of S15 gene and measured using the equation $2^{(Ct\ S15 - Ct\ P53)}$. Error bars represent SD.

From the data plotted in Figure 27, AdGFP showed a significant difference in DNA level between the cases. Although AdGFP obtained the highest DNA level in all cases (A-E) at 72 hours post-infection, this increase was insignificant at p values >0.05 (one-way ANOVA). Viral DNA replication in the case of the plasmid B was significantly higher than the negative control (F) at p values <0.05 (Student t test), although p53 expression pattern of plasmid B was almost the same compared to other plasmids. Different peaks that show the maximum p53 expression were obtained at different time points. When the data were compared to the previous results in H1299, AdGFP showed greater replication efficiency, as it reached a similar Ad5wt level but to a lesser extent.

Determination of Ad5dlE1b55kDa replication efficiency

Ad5dlE1b55kDa DNA level in HeLa cells was significantly lower than Ad5wt and AdGFP. As shown in Figure 28, the highest level was reached at 2 hours post-infection and it sharply reduced after this point reaching either the basal level or zero at 72 hours post-infection. No significant difference of viral DNA level per cell was found under all cases (A-F) that express exogenous (A-E) and endogenous (F) p53. The level of p53 expression in all plasmids was almost the same with insignificant difference at all time post-infection.

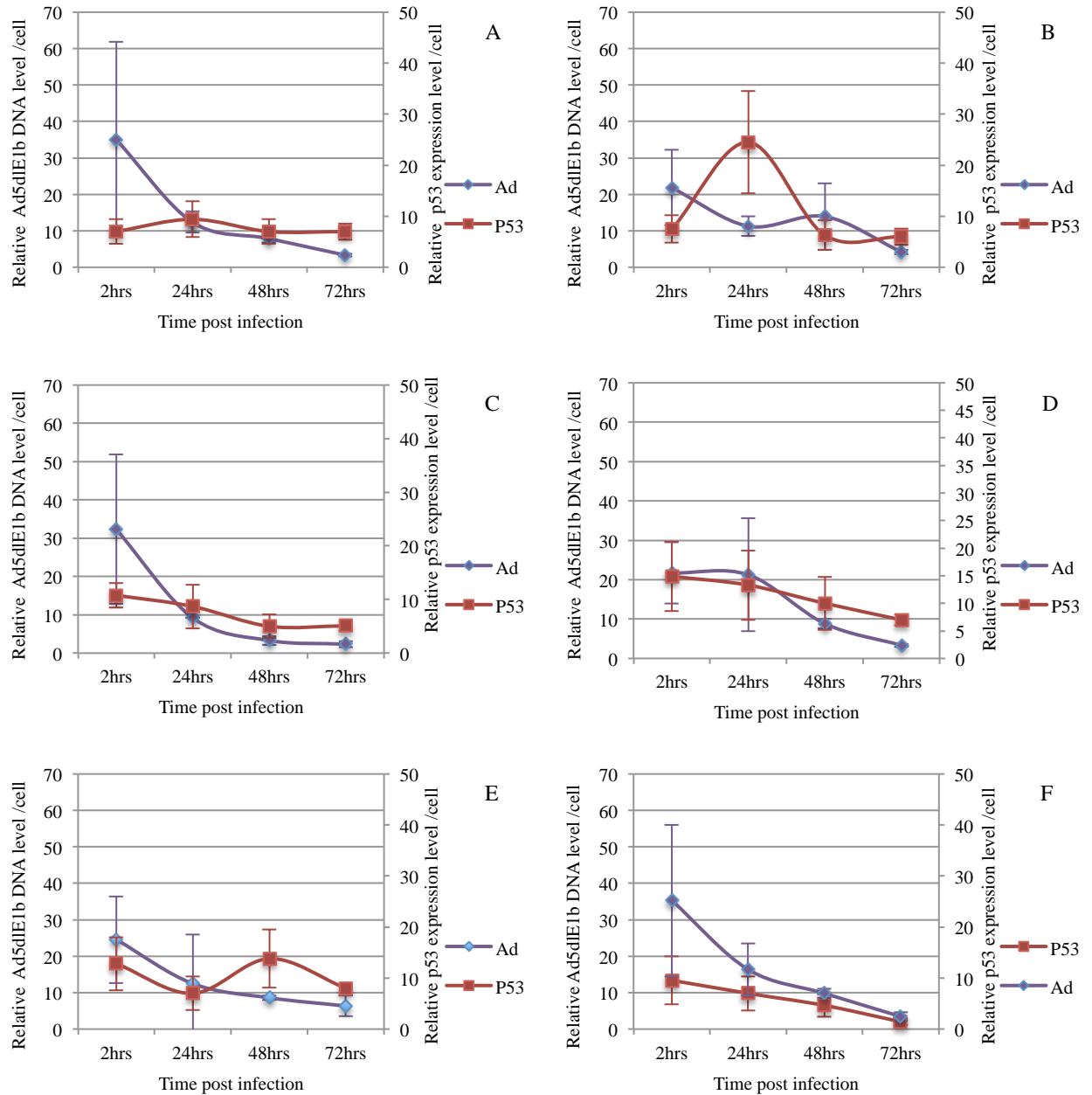


Figure 28: Effect of p53 levels on Ad5dlE1b replication efficiency in HeLa cells. Relative Ad5dlE1b DNA levels in different p53 expression levels that obtained from HeLa cells transfected with plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with 5S using the equation $2^{(Ct\ 5S - Ct\ hexon)}$. Relative p53 expression levels were normalized using the expression of S15 gene and measured using the equation $2^{(Ct\ S15 - Ct\ P53)}$. Error bars represent SD.

Comparison and characterization of Ad5wt, AdGFP and Ad5dlE1b55kDa growth in H1299 and HeLa cells

To compare viral growth kinetics of Ad5wt, AdGFP and Ad5dlE1b55kDa in relation to different p53 levels, the same above experiment data were plotted and expressed in a different way for comparison purposes. The results represent the replication phenotype of each virus, which was characterized in both H1299 and HeLa cell lines expressing exogenous p53 from p53 expression vectors and also in matched cell lines without exogenous p53 expression.

Ad5wt growth in H1299 and HeLa cells

From the results, there was insignificant difference in viral growth ability of Ad5wt in both H1299 and HeLa cells with or without respect to p53 expression at all times post-infection (Student t test, p values >0.05). The viral DNA replication was greatest at 72 hours post-infection (Figure 29).

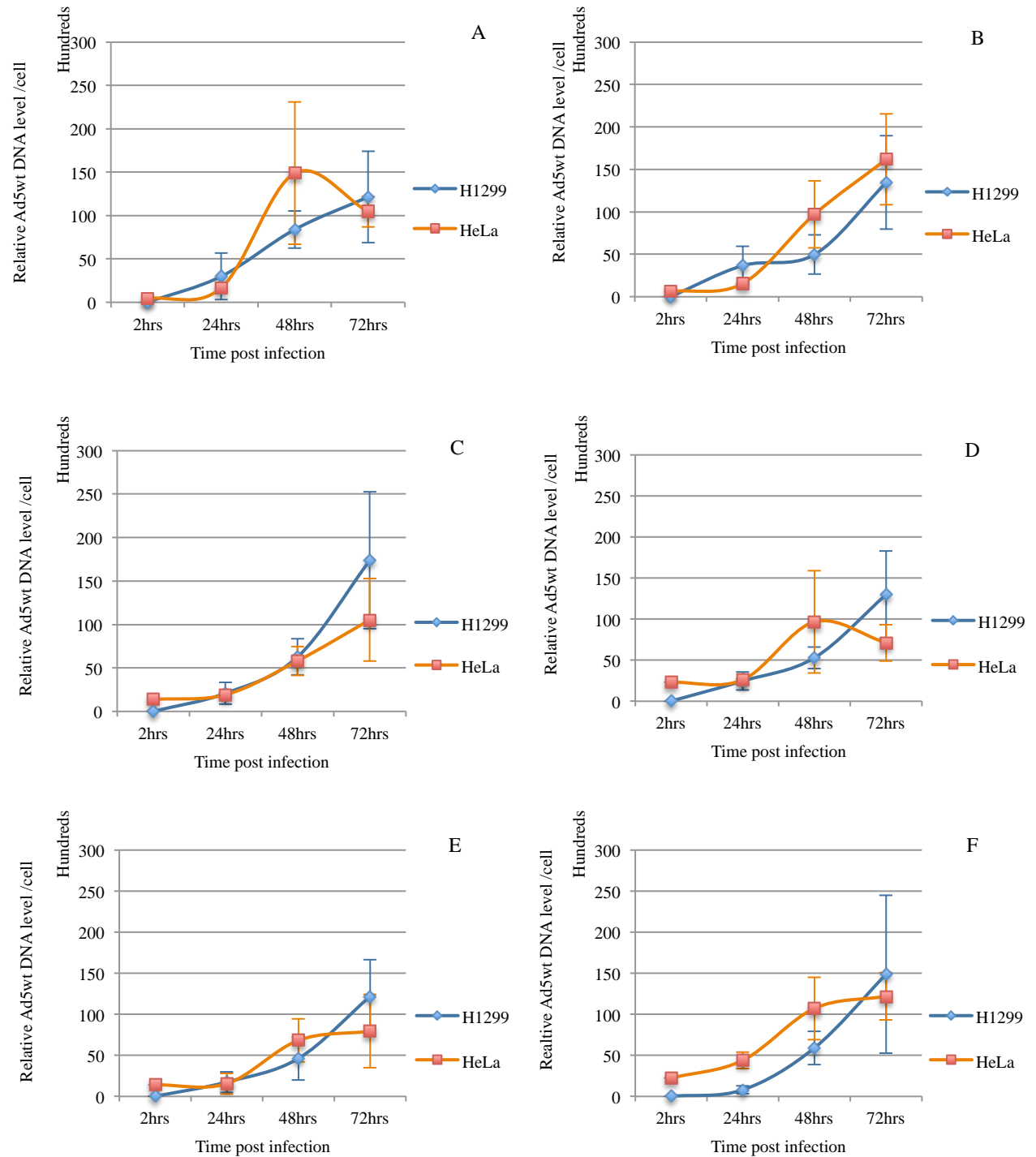


Figure 29: Ad5wt growth in H1299 and HeLa cells. Relative Ad5wt DNA levels in different p53 expression levels that obtained from both cell lines transfected with plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with housekeeping genes using the equation $2^{(Ct \text{ cellular gene} - Ct \text{ hexon})}$. Error bars represent SD.

AdGFP growth in H1299 and HeLa cells

AdGFP growth kinetics differed in H1299 and HeLa. A significant viral DNA level at various degrees was observed in HeLa cells compared to H1299 cells at all times post-infection (Student t test, p values <0.05). The viral DNA replication was started at 24 hours post-infection until it reached its peak at 72 hours post-infection, the highest replication point (Figure 30).

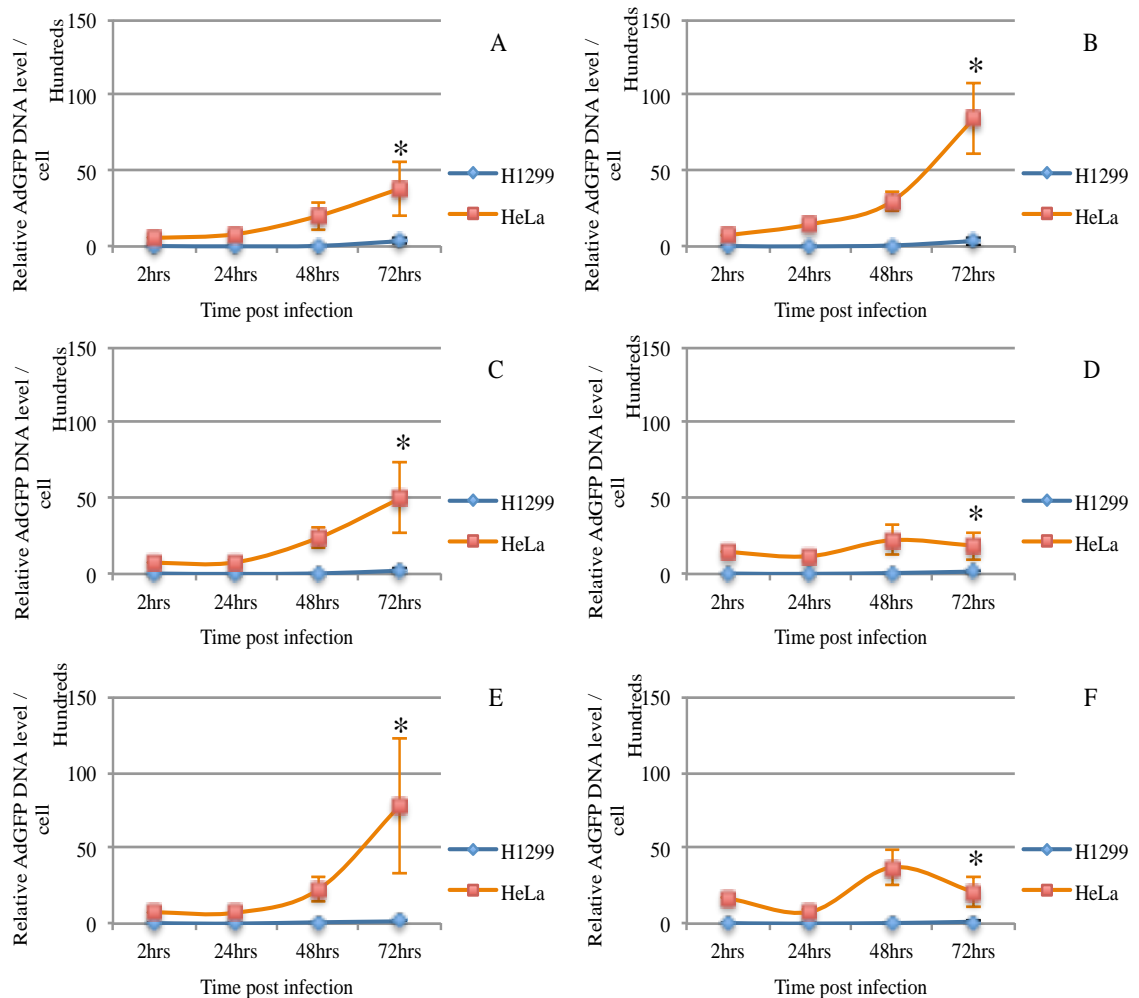


Figure 30: AdGFP growth in H1299 and HeLa cells. Relative AdGFP DNA levels in different p53 expression levels that obtained from both cell lines transfected with plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with cellular gene using the equation $2^{-(Ct \text{ cellular gene} - Ct \text{ hexon})}$. Error bars represent SD.

Ad5dlE1b55kDa growth in H1299 and HeLa cells

The growth kinetics of Ad5dlE1b55kDa in different cell lines was different, as the viral DNA level of Ad5dlE1b55kDa in H1299 was higher than HeLa reaching a significant p value level of <0.05 (Student t test) under all plasmid conditions at 48 and 72 hours post-infection (Figure 31). The replication of viral DNA reached its maximum at 72 hours post-infection.

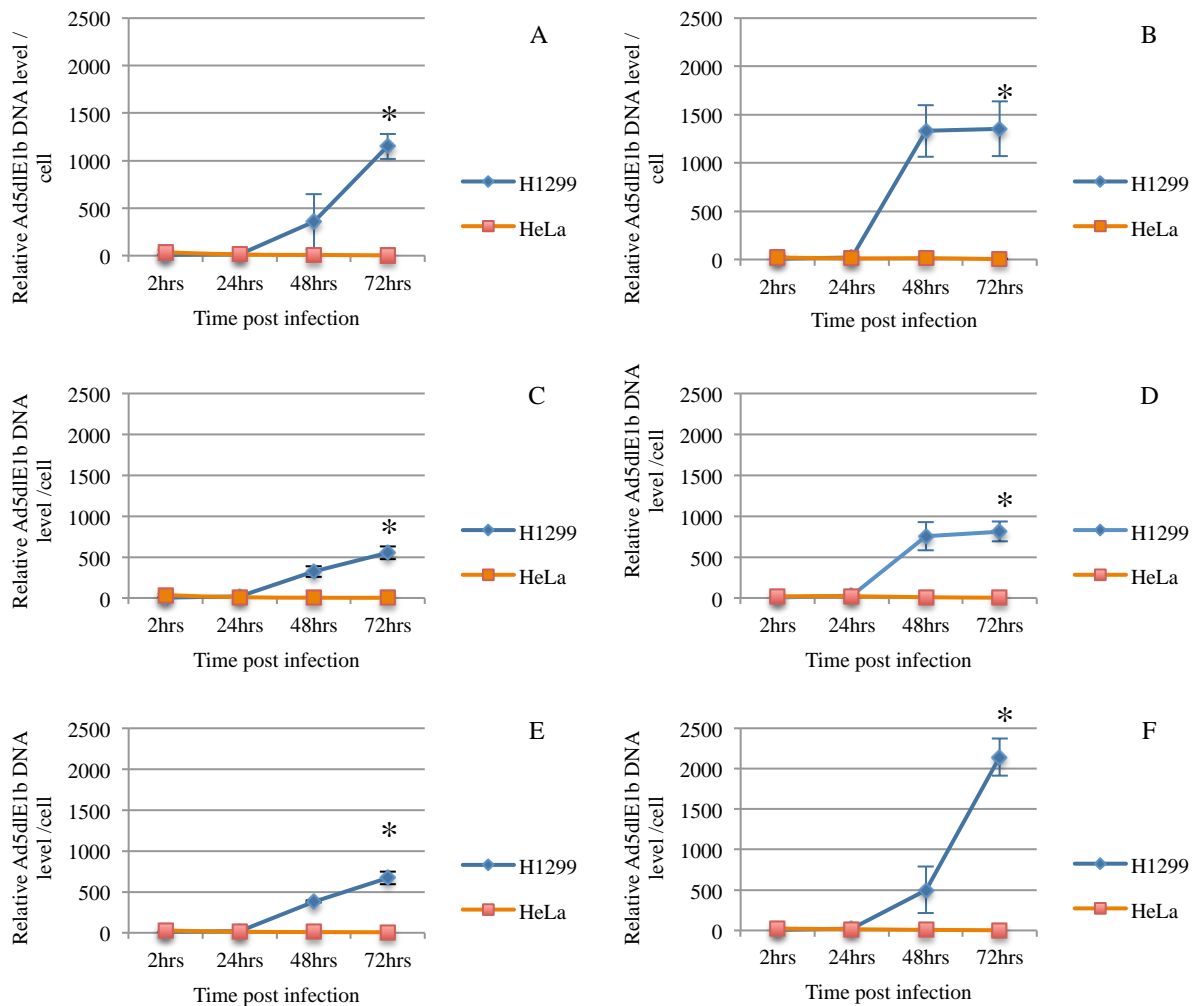


Figure 31: Ad5dlE1b growth in H1299 and HeLa cells. Relative Ad5dlE1b DNA levels in different p53 expression levels that obtained from both cell lines transfected with plasmids (A, B, C, D, and E) and (F serve as PUC19 negative control) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with a cellular gene using the equation $2^{-(Ct \text{ cellular gene} - Ct \text{ hexon})}$. Error bars represent SD.

EVALUATING THE IMPACT OF P53 FUNCTIONAL STATUS ON THE REPLICATION EFFICIENCY OF ONCOLYTIC AD5dlE1b MUTANT

To test whether the replication efficiency of oncolytic Ad5dlE1b55kDa mutant was dependent on the functional activity of p53, the growth of Ad5dlE1b55kDa was determined in cells expressing various p53 forms, wild type and mutants. Since the existence of such mutations influences the activity of p53 at various degrees (depending on the mutation site), we constructed multiple mutant p53 plasmids that express various mutant forms of p53 along with a wild type p53 plasmid to generate matched pair p53-expressing cells. The introduced mutations were chosen based on a bioinformatics analysis of cancer-associated p53 mutation sites. Then, we evaluated the growth of Ad5dlE1b55kDa in various mutant-p53 transfected cells. Lastly, we investigated the functional status of mutant p53 that rescued viral growth as well as determined the viral rescuing mechanism underlying tumor selectivity by transcriptome profiling.

The H1299 (p53-null) cell line was chosen based on literature to perform a fair and unbiased comparison. It was transiently transfected with the engineered plasmids that expressing wild type and mutant p53 versions along with the controls (mock transfected) and then infected with Ad5wt and Ad5dlE1b55kDa at day 1 post-transfection. Viral MOI and infection conditions were optimized for little variation and high consistency. Viral DNA level was determined as an actual indicator of viral replication efficiency at various times post-infection.

Bioinformatics analysis of p53 mutation sites

Since more than 90% of cancer-related p53 mutations are found distributed within the DNA binding domain affecting its stability and DNA binding ability as well as protein conformation, in this study we chose different mutations within DBD and PRD that may affect the function of the p53 protein differently to ensure diversity in the functional impact. The process of choosing the mutations was actually based on literature reviews and bioinformatics analysis of each mutation individually (Petitjean *et al.*, 2007). Missense mutation at codons 72, 175 and 268 were chosen for the study's purpose (Figure 32 & Table 10).

Mutant R72P locates in the PRD, which is required for apoptosis and growth suppression, was chosen based on a common polymorphism where amino acid 72 is arginine (R) instead of proline (P). P53 arginine is a more potent inducer of apoptosis and occurred with more frequency compared to p53 proline (Dumont *et al.*, 2003; Salvioli *et al.*, 2005). When p53 has an arginine at this position, it can modify the mutant protein's ability to bind and interact with other proteins such as MDM2 and p73, a p53-like protein inhibiting their function and also affecting the response to therapies (Dornan *et al.*, 2003).

Mutant R175H (arginine to histidine) is structural mutant that belongs to class I mutants and locates in the L2 loop of structural DNA binding motif p53. This missense point mutation substitution alters the conformational state of the p53 protein, yielding indirect disruption of DNA-binding ability (Cho *et al.*, 1994). It possesses critical function in stabilizing the loops that are involved in DNA binding. R175H is the third most frequently mutated residue associated with human cancer.

Mutant N268D (asparagine to aspartic acid) is a contact mutant that belongs to class II mutants and locates in the DBD. It is the most stable missense point mutation substitution that can occur in cancer (Petitjean *et al.*, 2007). It acts on different regions of the core domain of p53 affecting its stability and its DNA binding activity resulting in hindring the tumor suppressor functions, the most important of which are the cell-cycle arrest and apoptosis (Cho *et al.*, 1994).

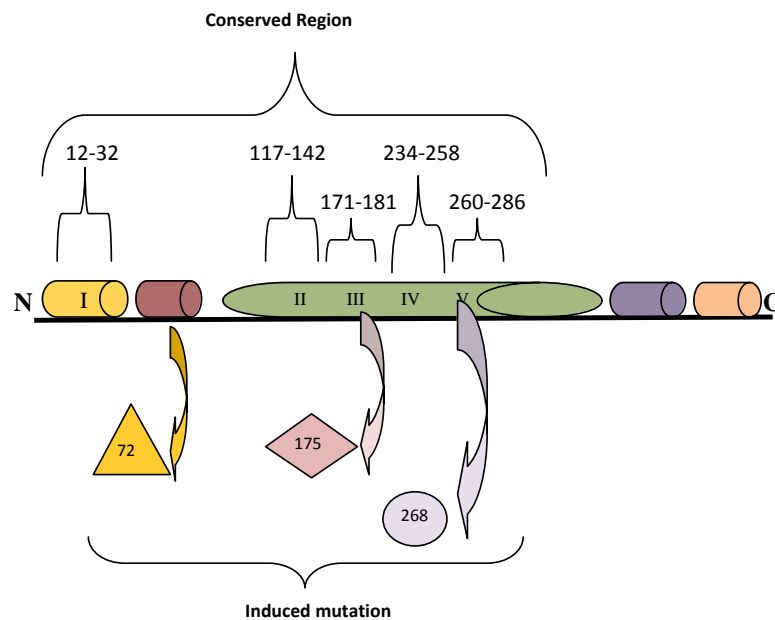


Figure 32: The chosen p53 mutations in this study (Modified from May and May, 1999).

Table 10: Mutations codons, amino acid substitutions, and locations.

Mutations	Mutated codon	Mutated amino acid	Location
Residue 72	CCC→ CGC	Pro → Arg	PRD
Residue 175	CGC→ CAC	Arg → His	DBD
Residue 268	AAC → GAC	Asn → Asp	DBD

Construction of mutant p53 plasmids

The construction of multiple mutant p53 vectors was achieved using a similar cloning strategy in all engineered plasmids. HPRE plasmid (pD) from phase I was utilized in this chapter as the backbone plasmid for all the constructs which share the same promoter and cis-acting elements but differ in the p53 gene sequence. The overall cloning strategy is shown in Figure 33.

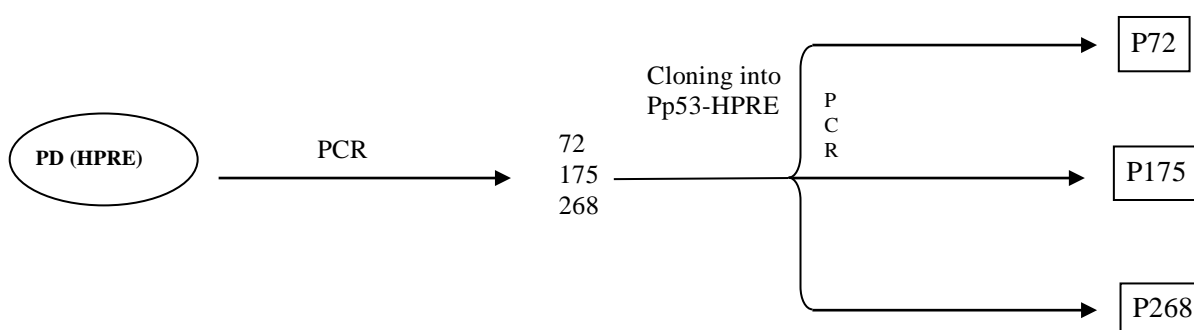


Figure 33: Schematic of overall cloning strategy of mutant p53 plasmids

Generation of mutations in p53 coding sequence

Site directed mutagenesis method

The introduction of R175H was achieved through the use of a site directed mutagenesis approach along with the mega primer strategy (Tyagi *et al*, 2004). In this method, two rounds of PCRs were conducted utilizing two external oligonucleotide primers and one internal mutagenic primer to obtain the required mutated fragment. HPRE plasmid (pD) was used as a template for the first round of PCR reaction since it contains the p53 wild type sequence that needs to be mutated. Two primers, in which one

of them is the internal mutagenic primer, were used to generate the first PCR product that contains the desired mutation. Then, a second round of PCR reaction was performed on the first PCR product using the intact external primers (flanked with unique restriction enzymes for cloning purposes) to amplify a plentiful amount of mutated p53 fragment (Figure 34).

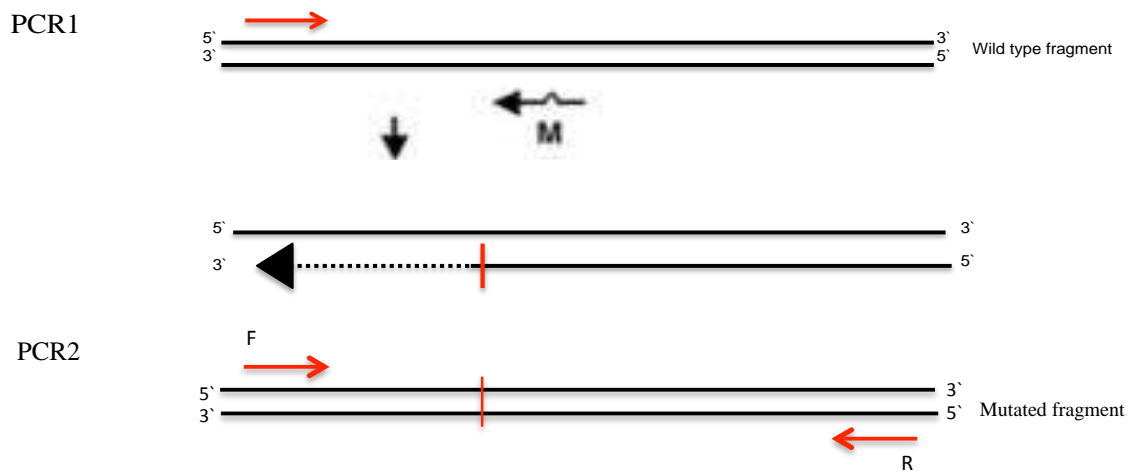


Figure 34: Site directed mutagenesis approach with mega primer polymerase chain reaction technique (Modified from Tyagi *et al.*, 2004)

Construction of p53-P72R plasmid

The plasmid contains mutation in the p53 sequence at codon 72 in which proline is substituted with arginine (CCC → C^GC). This mutated fragment was achieved by annealing of two mutated oligonucleotides. It is also flanked with unique restriction enzymes (the closest to the mutation site) to serve the cloning into HPRE plasmid as a replacement of the wild type p53 sequence as shown in Figure 35.

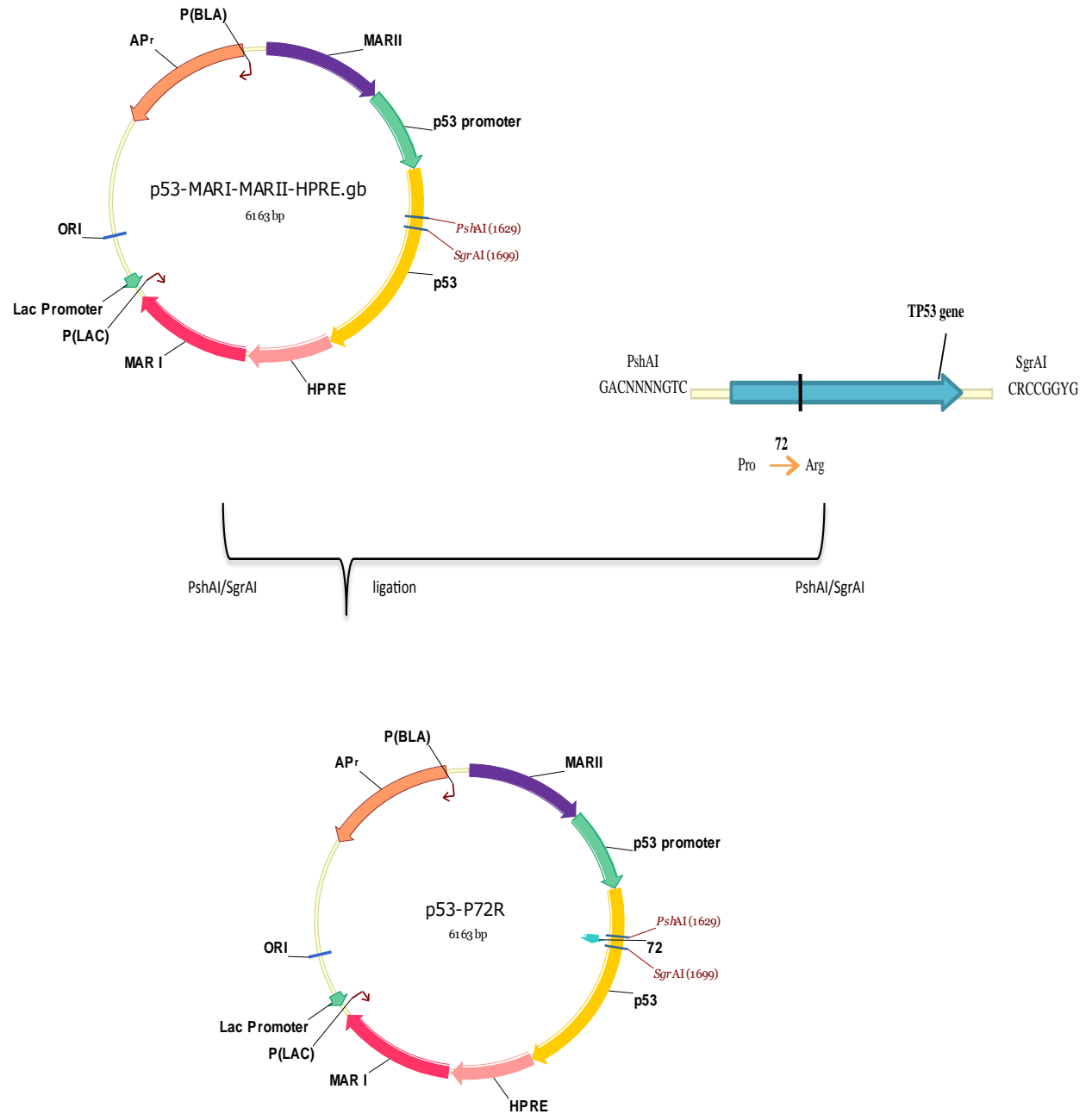


Figure 35: Schematic diagram showing the strategy for constructing the p53-P72R plasmid containing a mutation at codon 72 of p53 coding region. Mutant fragment was generated through annealing of two mutated oligonucleotides.

The final plasmid was then confirmed by restriction enzyme digestion (Table 11 & Figure 36) and sequencing analysis was also performed to confirm the presence of the desired mutation at the desired site as shown in Figure 37.

Table 11: Restriction enzyme analysis of p53-P72R plasmid

Lane number	Enzyme	Expected band size (bp)
1&2	BamHI	4129, 2038

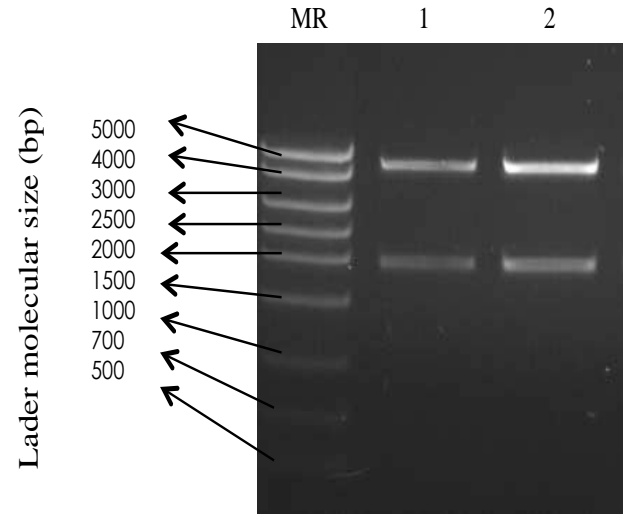


Figure 36: Restriction enzyme confirmation for p53-P72R plasmid. Restriction enzyme digestion of p53-P72R plasmid on agarose gel. MR: Norgen's MidRanger ladder.

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gaccaggtccagatgaagctcccagaatgccagaggctgctcccCGCgtggcccctgcaccagcagctcctaca
ccggcg
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Figure 37: Confirmation of full-length sequence of p53-P72R fragment by sequencing analysis (n=3) to ensure 100% sequence accuracy and the presence of the desired mutation.

Construction of p53-R175H plasmid

This plasmid contained a point mutation at codon 175 that changed the amino acid from arginine to histidine (CGC→CAC). This mutant form of p53 was accomplished by using site-directed mutagenesis as described above. The flanking restriction enzymes at the two ends of the fragment facilitate the cloning into the HPRE plasmid as a replacement of wild type p53 sequence (Figure 38).

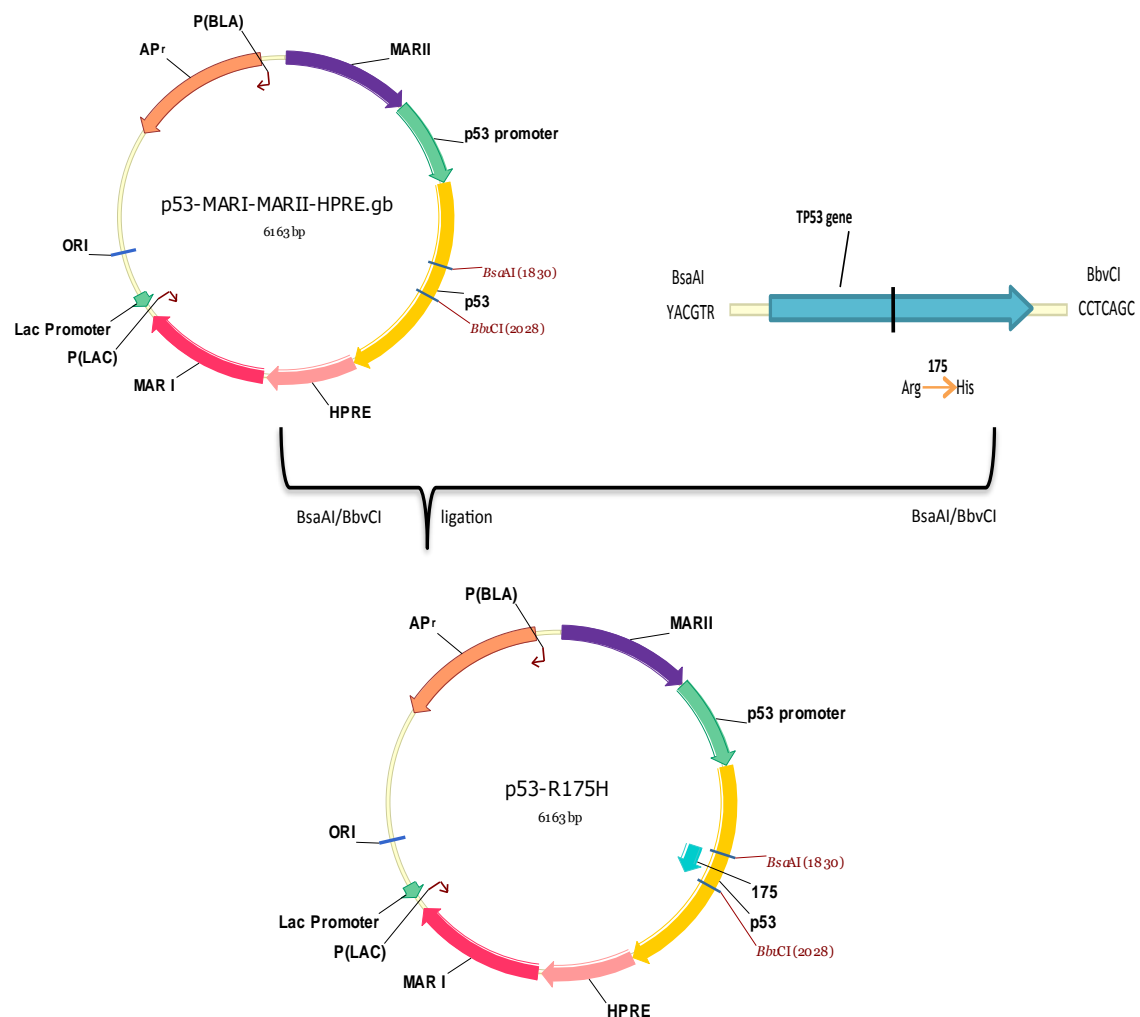


Figure 38: Schematic diagram showing the strategy for constructing the p53-R175H plasmid containing a mutation at codon 175 of p53 coding region. Mutant fragment was generated through site directed mutagenesis approach.

The final confirmation of digested plasmid is shown in Table 12 & Figure 39 and sequenced to determine which allele the mutation resided on (Figure 40).

Table 12: Restriction enzyme analysis of p53-R175H plasmid

Lane number	Enzyme	Expected band size (bp)
1	SfoI	6167
2&3	BamHI	4129, 2038

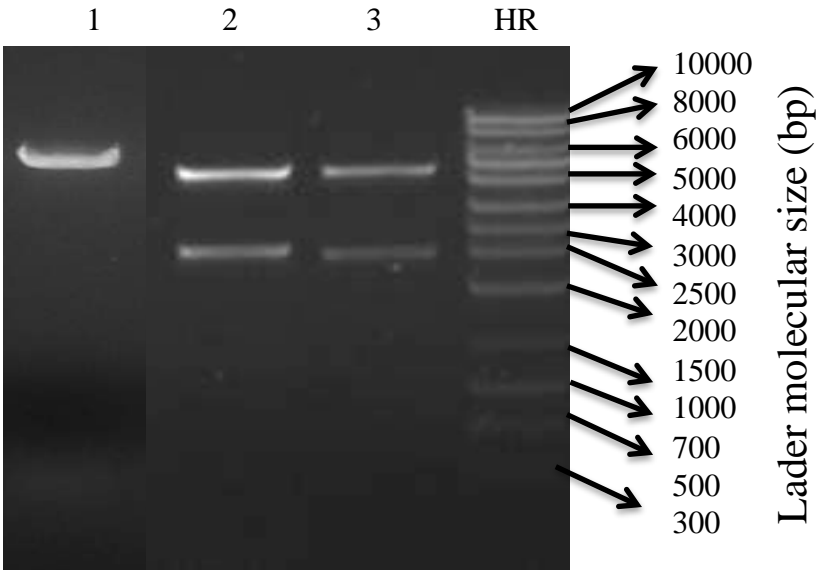


Figure 39: Restriction enzyme confirmation for p53-R175H plasmid. Restriction enzyme digestion of p53- R175H plasmid on agarose gel. HR: Norgen’s HighRanger ladder.

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cacgtactccctgccctcaacaagatgtttgccaaactggccaagacctgccctgtgcagctgtgggttgattccacacc
ccgcccggcacccgcgtccgcgccatggccatctacaagcagtcacagcacatgacggagggtgtgaggcgctgcccc
caccatgagCACtgcctcagatagcgatggtctgccccctcctcage
```

Figure 40: Confirmation of full-length sequence of p53-R175H fragment by sequencing analysis (n=3) to ensure 100% sequence accuracy and the presence of the desired mutation.

Construction of p53-N268D plasmid

The plasmid includes a mutation at 268 spot in p53 sequence. This mutation converted the asparagine amino acid into the aspartic acid (AAC → GAC). The gene-constructed fragment was flanked at both ends with the nearest restriction enzymes to the mutation site. These restriction sites are essential in cloning this fragment into the HPRE plasmid to obtain the final p53-N268D plasmid. The construction strategy is shown in Figure 41.

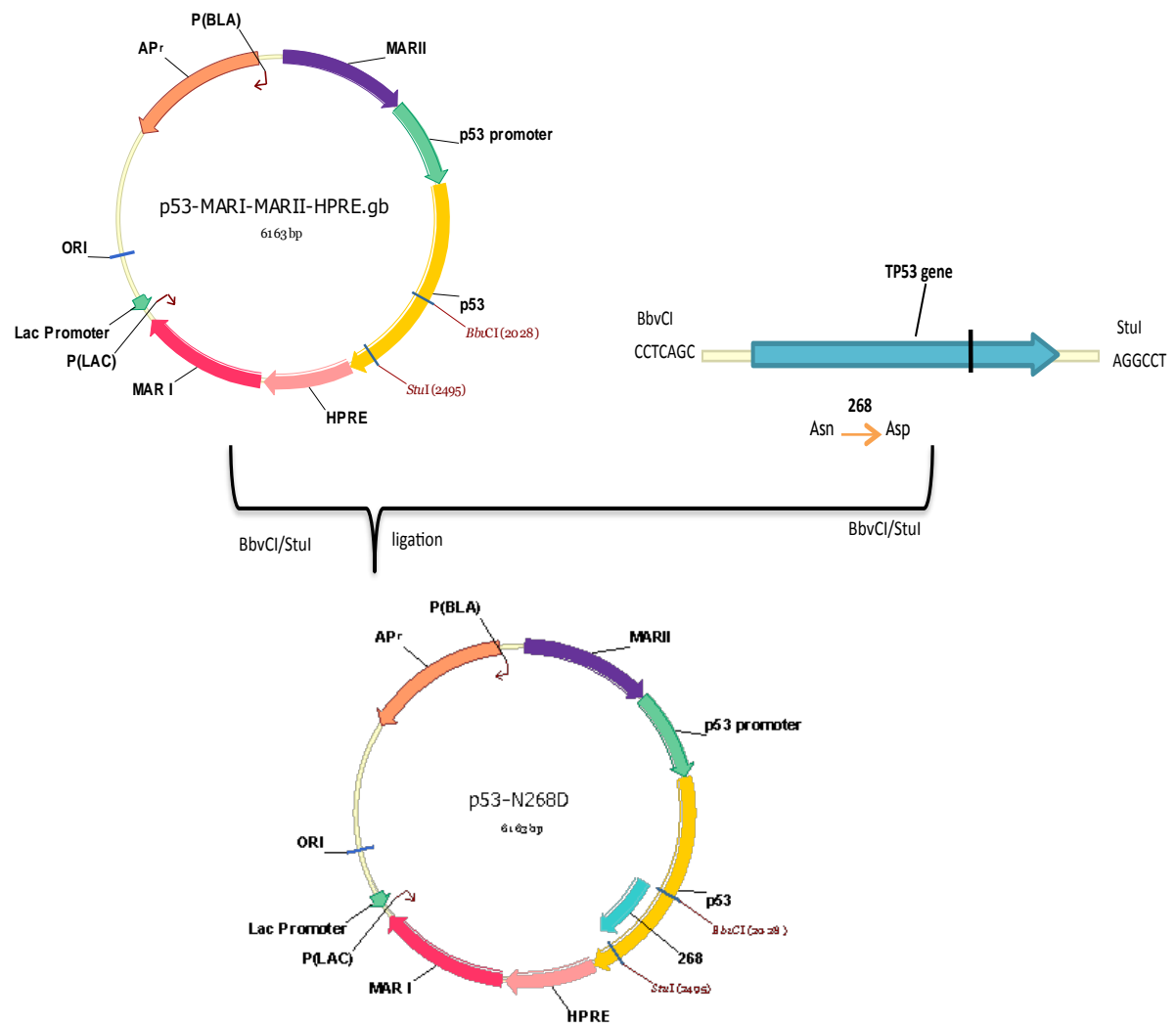


Figure 41: Schematic diagram showing the strategy for constructing the p53-N268D plasmid containing a mutation at codon 268 of p53 coding region. Mutant fragment was obtained from gene construction.

As shown in the gel (Figure 42), when the plasmid was digested with a restriction enzyme (Table 13), the observed bands were similar to what is expected. Also, the sequencing analysis showed the right sequence as required (Figure 43).

Table 13: Restriction enzyme analysis of p53-N268D plasmid

Lane number	Enzyme	Expected band size (bp)
1	Scil/NdeI	5629, 538

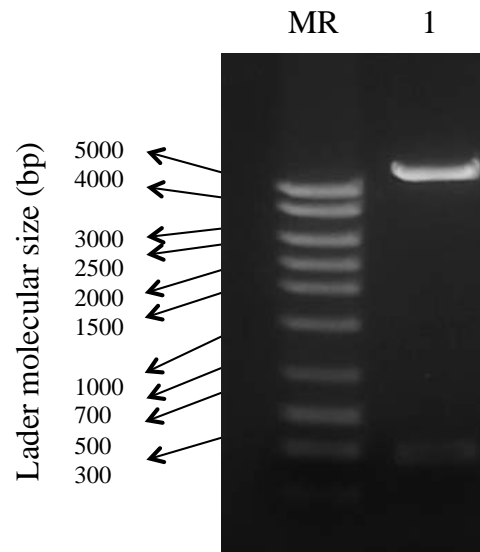


Figure 42: Restriction enzyme confirmation for p53-N268D plasmid. Restriction enzyme digestion of p53-N268D plasmid on agarose gel. MR: Norgen's MidRanger ladder.

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Cctcagcatcttatccgagtggaaggaaatttgcgtgtggagtatttggatgacagaaacacttttcgacatagtgtggtggt
gccctatgagccgcctgaggttggtctgactgtaccaccatccactacaactacatgtgtaacagttcctgcatggcgccg
atgaaccggaggcccatcctcaccatcatcacactggaagactccagtggtaatctactgggacggGACagctttgagg
tgcgtgtttgtgcctgtcctgggagagaccggcgacagaggaagagaatctccgcaagaaaggggagcctcaccacg
agctgccccagggagcactaagcgagcactgcccacaacaccagctcctctccccagccaaagaagaaccactgg
atggagaatatttcacccttcagatccgtgggcgtgagcgcttcgagatgttccgagagctgaatgaggcct

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Figure 43: Confirmation of full-length sequence of p53-N268D fragment by sequencing analysis (n=3) to ensure 100% sequence accuracy and the presence of the desired mutation.

Characterization Ad5 dlE1b55kDa growth in cells expressing mutant p53

To examine the effect of p53 functional activity on the replication efficiency of oncolytic Ad5dlE1b55kDa, the viral growth was examined under various mutants p53 backgrounds assuming that each mutant transcript should differ functionally from the others. The previously engineered mutants and wild type p53 constructs (Table 14) were used to transfect H1299 cells generating different versions of wild type or mutant p53-expressing cells. The reason for choosing these cells was the fact that it is a p53-null cell line, which serves the desired purpose. Two negative controls were included in the experiment: PUC19 plasmid and mock transfected cells. In addition, infection with wild type Ad (Ad5wt) was used as a control beside Ad5dlE1b55kDa (Ad5dlE1b) (Table 14). Briefly, H1299 cells' monolayer was transfected with test and negative control plasmids. Transfection with plasmid conditions from G to L was conducted in triplicate in 24-well plate using Lipofectamine 2000 reagent and 6 hours post-transfection the medium was replaced. Twenty-four hours post-transfection, viral infection of Ad5wt and Ad5dlE1b55kDa was carried out at MOI of 10 PFU/cell. Samples were then collected at different times post-infection and total DNA along with total RNA were isolated for further analysis.

Table 14: List of adenoviruses and mutant p53 plasmids used in this study

Virus	Description	Source	Reference
Ad5dlE1b	dl E1b55kDa + dl E3	Dr. Haj-Ahmad	(Haj-Ahmad, 1986)
Ad5wt	dl E3	Dr. Haj-Ahmad	(Haj-Ahmad, 1986)
Plasmid abbreviation	Plasmid name	P53 genotype	Figures
G	pHPRE	Wild type p53	Figure 14
H	p53-P72R	Mutant p53 (P72R)	Figure 35
I	p53-R175H	Mutant p53 (R175H)	Figure 38
J	p53-N268D	Mutant p53 (N268D)	Figure 41
K	PUC19	Negative control	-
L	-	Negative control	-

Ad5wt and Ad5dlE1b55kDa replication in mutant p53-transfected H1299

Relative viral DNA level was used as a determinant of viral replication efficiency. Equal amount of isolated DNA samples from Ad5wt-infected cells and Ad5dlE1b55kDa-infected cells were used as templates for TaqMan qPCR reaction. Specific adenovirus probe and primers were used to amplify the hexon gene. The obtained C_{Ts} were used to perform ΔC_T threshold method to calculate viral DNA level per cell after normalization with GAPDH, a housekeeping gene. The measurements were done through the equation $2^{(C_t \text{ GAPDH} - C_t \text{ hexon})}$. The data were analyzed using a one-way ANOVA test. P values <0.05 were considered significant.

From the data in Figure 44, Ad5wt showed significantly higher viral DNA levels than Ad5dlE1b55kDa over various times points post-infection. Both viruses reached the highest growth level at 72 hour post-infection. In the case of Ad5wt, there was insignificant difference in the viral DNA levels under this window (G, H, I, K and L) (one-way ANOVA, p values >0.05) although it showed a slightly higher level under mutant p53N268D (one-way ANOVA, p values <0.05). On the other hand, Ad5dlE1b55kDa reached the highest DNA level under mutant p53R175H with significant difference compared to other conditions (one-way ANOVA, p values <0.05). However, viral DNA levels that obtained from the other mutants showed the same results compared to the controls (one-way ANOVA, p values >0.05) whereas viral replication under wild type p53 was the lowest among the others. It is important to note that p53 expression level was measured from the isolated RNA that was obtained from the same single sample and optimized for long-lived expression. Viral DNA levels of both viruses

under all conditions did not show strong correlation with the level of p53 expression as was concluded in the previous chapter (Data not shown).

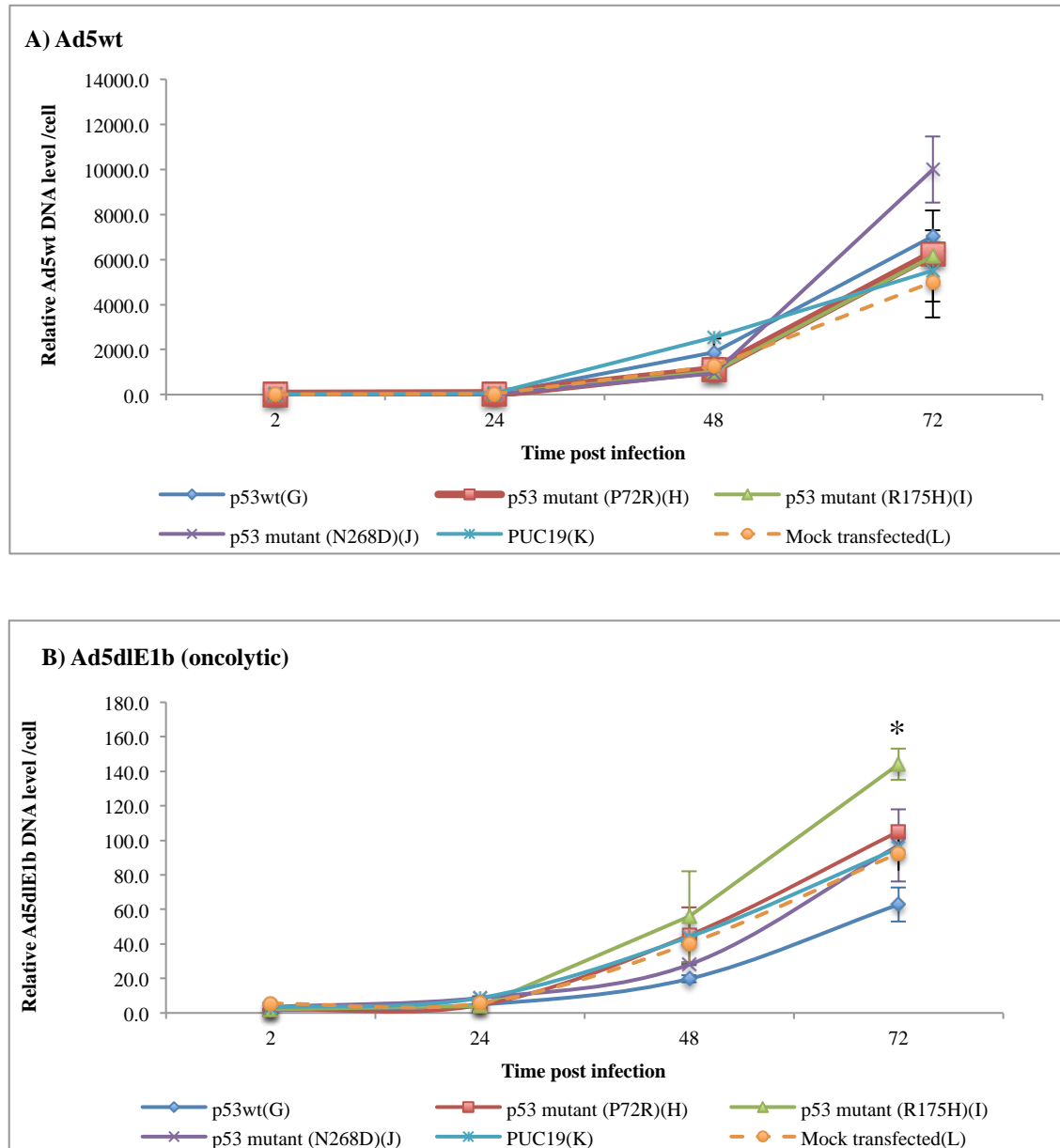


Figure 44: Adenovirus replication in H1299 cells expressing various p53 mutants. A) Relative Ad5wt DNA levels in wild type p53 (G) and different mutants p53 (H, I and J) (K and L serves as PUC19 and mock transfected negative controls) (n=3) over 2, 24, 48 and 72 hours post-infection. B) Relative AdE1b 55kDadeleted DNA levels in wild type p53 (G) and different mutant p53 (H, I and J) (K and L serve as PUC19 and mock transfected controls) (n=3) over 2, 24, 48 and 72 hours post-infection. Relative DNA levels were measured after normalization with GAPDH using the equation $2^{-(Ct\text{ GAPDH} - Ct\text{ hexon})}$. Error bars represent SD.

Table 15: Relative amount of viral DNA in H1299 cell line at 2 hours and 72 hours post-infection (replication ratio) in correlation with p53 status.

A) Ad5wt					
P53 genotype	P53 status	Viral DNA copies/cell at 2 hours	Viral DNA copies/cell at 72 hours	Replication Ratio 72/2 hours	CPE
Wild type	wt	1.0	7035	7147	+++
mutated	P72R	1.2	6254	5272	+++
mutated	R175H	1.1	6154	5612	++
mutated	N268D	2.6	9988	3897	+++
-	-	1.0	5515	5720	++
B) Ad5dlE1b55kDa					
Wild type	wt	1.5	62.80	41.9	-
mutated	P72R	1.9	105.1	55.3	+
mutated	R175H	2.1	144.0	68.6	++
mutated	N268D	3.5	97.10	27.7	+
-	-	3.0	95.50	31.4	+

-Grade of visual cytopathic effect at 72 hours post-infection (the highest virus copy number). (- no CPE, + weak CPE, ++ moderate CPE, +++ strong CPE)

From the results in Figure 44, we found that mutant p53R175H successfully rescued Ad5dlE1b55kDa growth more than the other mutants in the study. It is assumed that this mutation functionally inactivated p53; therefore it supported the replication of oncolytic Ad5dlE1b55kDa mutant. Further investigation of the functional activity of this mutation is essential to reveal the nature of the relationship between oncolytic E1b deleted adenovirus replication efficiency and p53 status during infection in the context of cancer virotherapy.

Microarray- based gene expression profiling

Mutant p53 (p53R175H) that showed successful rescuing of oncolytic Ad5dlE1b55kDa was further investigated at the transcriptional level to examine its functional activity. This was achieved by comparing gene expression profiles of both mutant p53 and wild type p53 in the context of p53 target genes that mediate its function. In this section, we also determined the rescuing mechanism of tumor selectivity of oncolytic Ad5dlE1b55kDa in the same context and revealed how this mutant p53R175H affected the cellular transcriptome and modulated Ad5dlE1b55kDa replication. This was accomplished by comparing the expression profiling of wild type p53 and mutant p53 samples but after the infection with Ad5dlE1b55kDa to assess the relative importance of p53 target genes in mutant p53 transcript, a physiologic target of E1b55kDa deleted adenovirus.

The same above experiment with Ad5dlE1b55kDa infection was conducted again and p53 expression was optimized for long-lived expression as well. In this study, we chose to control the cellular difference, infectibility, and the heterogeneity of p53 induction by selecting one cell line to be the control cell line and a model system, with which we can perform the comparisons. This cell line is H1299 (p53-null) cell line.

Microarray-based gene expression analysis was performed on the purified and DNase-treated RNA samples. The concentration of total RNAs was determined and the quality of the samples was also analyzed using the Agilent RNA 6000 Kit. Five RNA samples were sent for gene expression profiling. The first two RNA samples were isolated from H1299 cells that were transiently transfected with wild type and mutant p53

plasmids (mock-infected samples). The second two RNA samples were isolated from H1299 cells that were transiently transfected with wild type and mutant p53 plasmids and infected afterwards with Ad5dlE1b55kDa at day one post-transfection (infected samples) at MOI of 10 PFU/cell. The fifth RNA sample, which was incorporated in the comparison in order to obtain a meaningful result, was H1299 RNA cellular background and served as a control sample. The time point 72 hours post infection was chosen to examine the expression pattern of cellular genes because p53 gene expression and viral DNA replication efficiency were the highest at this time point.

The transcriptome expression analysis of all RNA samples was performed using Affymetrix Genechip Human transcriptome array 2.0 (HTA 2.0). This platform provides gene expression profiling of all known transcripts. The GeneChip system is comprised of 25-mer oligonucleotide probes synthesized on quartz chips and assembled into cartridges. The p53-dependent gene expression patterns of the four samples were analyzed using Transcriptome Analysis Console software to categorize the significant differentially expressed genes that are biologically meaningful. These genes should satisfy the modest level of statistical significance (Student t test p-values <0.05) and ranked by minimum fold-change cutoff of 2.0, which was calculated by dividing the signal intensity of each gene mRNA against the corresponding mRNA level of the control.

More than fifty three thousand protein-coding genes and non-coding RNAs including the internal genes and controls were analyzed in all samples. Generally, between 500 and 800 genes showed up or down regulation among the tested samples. Since p53 target genes were the focus of this study, they were highlighted and analyzed

for their expression profile in the four samples. The basis of upregulation and downregulation of p53 target genes was based on a minimum fold change threshold. Two hundred and thirty-two target genes were analyzed for their expression. Of these genes, forty-two were found to share the same expression patterns among the four samples. Between the mock-infected samples, which are wild type p53 and mutant p53 transcripts, 82 genes were commonly expressed and 65 genes were differentially regulated. On the other hand, between the two infected samples, 107 genes shared the same expression patterns and 45 genes were differentially expressed. Most of these genes, if not all, mediate p53 functions including INSR, MDM2, PIDD, Bak, ZEB2, CASP1, CASP2, CCNB1, E2F4, and more which are distributed between cell cycle, apoptosis, DNA repair, cell proliferation and growth, cell division and stemness. The detailed results are shown in Table 16.

Table 16: Microarray gene expression profiles of up and down regulated genes in the test samples after normalization with control sample (H1299 RNA).

Action	# Of genes				Category	Reference	
	Mock infection		Viral infection				
	WTP53	MTP53	WTP53	MTP53			
Up regulation	369	303	439	475	Transcription, protein transport, gene expression, cell survival, cytokinesis, interferon pathway, pathways in cancer, stemness, mitochondrion distribution, organization, mitotic sister chromatid segregation, cell cycle, apoptosis, metabolism, immune response, oxidative stress, signal transduction, metabolic process, DNA repair, DNA replication, receptors, cell adhesion, cell growth, Damage response,	Data not shown	
Down regulation	202	267	413	428	As above	Data not shown	
P53 target genes (232)							
Commonly expressed among all (4 conditions)			42			Angiogenesis, cell cycle, DNA repair, Apoptosis, cell proliferation, stemness, metabolism, transcription, signal transduction targeted by p53, growth factors	Shown in Appendix
Commonly expressed (2 conditions)	82			107	Cell cycle, cell division, regulation of transcription, metabolism, DNA repair, apoptosis	Shown in Appendix	
NO change	85			80	DNA replication, Transcription, Pathways in cancer	Shown in Appendix	
Differentially expressed	65			45	Cell proliferation, apoptosis, cell division, cell growth and differentiation, DNA damage response, cell cycle regulation	Shown in Tables 17 & 18	

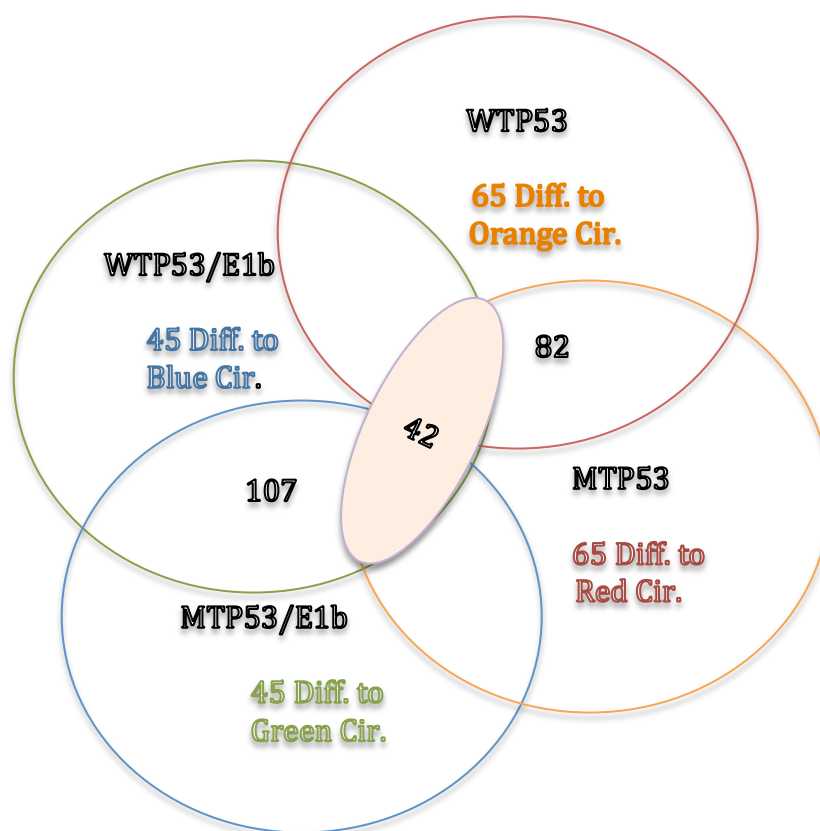


Figure 45: Venn diagram represents the commonly and differentially expressed genes in the four tested samples (wtp53, mtp53, E1b/wtp53, and E1b/mtp53).

Assessment of functional activity of mutant p53 that rescued oncolytic Ad5dlE1b55kDa mutant

In this part of the analysis the interest was in finding out the difference between the functional activity of wild type p53 and mutant p53. Again, since p53 is a transcriptional factor that regulates many genes in its pathway, p53 target genes that exert its function were highlighted throughout the study. Of note, p53 target genes were gathered and collected from International Agency for Research on Cancer (IARC), TP53 database, TP53 website, P53 books, human p53 signaling pathway array panels and P53-

pathway related literature reviews using PubMed (Zambetti, 2005; Petitjean *et al.*, 2007; Hainaut and Wiman, 2007; <http://p53.free.fr/>). The objective of this part was achieved when both mRNA transcripts of the two mock-infected samples were compared to each other after their normalization with the transcript of H1299 cellular background (control sample). Different kinetics of expression profiles of p53 target genes upon wild type and mutant p53 expression was obtained.

Of 232 genes, eighty-two genes were found to share the same expression patterns between mock-infected wild type p53 and mutant p53 transcripts (Figure 45). On the other hand, the rest were distributed between differentially regulated and no change. Sixty-five genes were found to differ in their expression patterns between mock-infected wild type and mock-infected mutant p53 transcripts including: Apaf1, PERP, MDM2, CDC7, ZEB2, CASP3, CASP2, BCL-2, E2F3, and more as shown in Table 17 and Figures 46 & 47 as well as Figure 49 that represents the volcano plot. These genes are distributed between cell cycle, apoptosis, DNA repair, cell proliferation and stemness.

In wild type p53 transcript, all genes that are responsible for mediating p53 functions upon expression and in response to unscheduled cell proliferation were returned back to their function to suppress tumor spread. On the other hand, the same genes but in mutant p53 transcripts were upregulated, which means that mutant p53 could not return to its normal function as tumor suppressor as wild type form. This indicates that gene expression profile was changed in a p53-dependent manner.

Table 17: P53 regulated genes in H1299 cells expressing wild type (wt) p53 and mutant (mt) p53. Grouping by functional category of genes whose average expression > or < 2 fold change. NC indicates no change.

Symbol Category	Affymetrx gene ID number	Gene Name	Specific/Other Categories	LOG ₂ fold change	
				Wtp53 /Mock	MtP53 /Mock
<i>Apoptosis</i>					
AXL	16862439	AXL receptor tyrosine kinase	Negative regulation of apoptotic process	-1	1.04
Apaf1	16755542	apoptotic peptidase activating factor 1	Intrinsic apoptotic signaling pathway	1.07	-1.01
Bak1	17018274	BCL2-antagonist/killer	Intrinsic mitochondrial apoptosis signaling pathway	1.03	NC
TRAF2	17091444	TNF receptor associated factor 2	Inhibitor of apoptotic process	NC	1.06
BCL-2	16855673	B-cell CLL/lymphoma 2	Blocks the apoptotic death of some cells	-1.13	1.03
TNFRS F10D	17075448	Tumor necrosis factor receptor superfamily member 10d	Inhibitory role in TRAIL-induced cell apoptosis	-1.02	NC
PIDD	16734119	P53 induced death domain protein	Apoptosis in response to DNA damage, effector of p53-dependent apoptosis.	1.04	-1
TP53I3	16895179	Tumor protein p53 inducible protein 3	Involved in p53-mediated cell death	1.17	NC
PERP	17024187	P53 apoptosis effector related to PMP-22	Apoptosis, p53 signaling pathway	-1.01	1.01
CASP3	16982011	caspase 3, apoptosis related cystine peptidase	Cleaves and activates caspases 6, 7 and 9	1.06	-1.09
CASP1	16743890	caspase 1, apoptosis related cystine peptidase	Regulation of apoptotic process-cell death-regulation of inflammatory response	1.13	-1.03
SIAH1	16826356	siah E3 ubiquitin protein ligase 1	implicated in induction of apoptosis	1.04	NC
SIAH2	16960517	siah E3 ubiquitin protein ligase 2	Negative regulator of apoptosis process	1.06	-1
SIAH3	16778790	siah E3 ubiquitin protein ligase 2	Ubiquitin-dependent protein catabolic process	1.09	NC

NFKB1	16969300	nuclear factor of kappa light polypeptide gene enhancer	Negative regulation of apoptotic process-positive regulation of transcription	NC	1.02
HK2	16881838	hexokinase 2	Apoptotic mitochondrial changes and transmembrane transport	-1.05	1.07
MCL1	16692775	bcl2 related/ myeloid cell leukemia sequence 1	Two transcripts one promote apoptosis and one inhibits apoptosis.	1.06	-1.07
PMAIP 1	16852683	phorbol-12-myristate 13 acetate induced protein 1	Positive regulation of apoptotic process- positive regulation of release of cytochrome c	1.01	-1.05
BBC3	16873675	Bcl-2 binding component 3	Induce mitochondrial dysfunction and caspase activation	-1.26	NC
BID	16932008	BH3 interacting domain death agonist	Mediator of mitochondrial damage induced by caspase-8	1.04	-1.05
CASP2	17052776	Caspase 2, apoptosis related cystine peptidase	Function in stress-induced cell death pathways, cell cycle maintenance, and the suppression of tumorigenesis	1.13	-1.03
Cell Proliferation					
KLF4	17096827	kruppel-like factor 4	Negative regulation of cell proliferation	-1.04	1.01
HDAC1	16662077	histone deacetylase 1	Control of cell proliferation and differentiation/ modulates p53 effect on cell growth and apoptosis.	1.12	-1.01
DNMT1	16868576	DNA (cytosine5) methyltransferase 1	Regulation of cell proliferation	NC	1.04
SFN	16661314	stratifin	Regulation of cell proliferation-intrinsic apoptotic signaling pathway	NC	1.02
TSC1	17099361	tuberous sclerosis 1	Regulation of cell cycle-regulation of cell proliferation	-1.01	1.03
IGF1	16769250	insulin-like growth factor 1	Positive regulation of cell proliferation	-1.15	1.01
ZEB2	17117888	zinc finger E-box binding homebox	DNA-binding transcriptional repressor and role in cell proliferation	-1.06	NC
Cell Cycle					
SESN1	17022362	Sestrin1	Cellular response to DNA damage and oxidative stress	NC	1.06

HMGA2	16753641	high mobility group AT-hook 2	P53 signaling pathway, regulation of cell cycle progression	1	-1.03
CHEK2	16933502	Checkpoint kinase 2	P53 signaling pathway, regulation of cell cycle progression	1.06	-1.02
BRCA2	16773840	Breast cancer 2	Cell cycle cytokinesis, negative regulation of mammary gland epithelial cell proliferation, nucleotide-excision repair	-1.02	1.38
CDC25 A	16953279	Cell division cycle 25A	Progression from G1 to the S phase of the cell cycle	-1.01	1.14
CDC7	16667037	Cell division cycle 7	Critical for the G1/S transition essential for initiation of DNA replication as cell division occurs	-1	1.17
CDC2	17016110	Cell division cycle 2	Essential for S phase	-1.01	1.05
E2F4	16820041	E2F transcription factor 4	Crucial role in the control of cell cycle and action of tumor suppressor protein	1.14	NC
E2F3	17005234	E2F transcription factor 4	Regulate the expression of genes involved in the cell cycle	NC	1.09
CDK6	17059756	Cyclin dependent kinase 6	Catalytic subunit of protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition.	-1.03	1.06
MLH1	16938899	Mutl homolog 1, colon cancer	Cell cycle	NC	1.08
ATR	16959985	Ataxia telangiectasia and Rad3 related	Negative regulation of DNA replication-cell cycle	-1.1	1.01
CCNA1	16774053	Cyclin A1	Regulate separate functions in Cell cycle, S phase and G2 phase	-1.12	1.05
CCND2	16746992	CyclinD2	Regulatory subunit of CDK4 or CDK6 kinases.	-1.09	1.04
CCNE1	16860418	CyclinE1	Regulator and mediate phosphorylation NAPT	-1.05	1.17
CCNG2	16968077	CyclinG2	Regulate cell cycle and cell division	-1.02	1.07
CCNB1	16985599	CyclinB1	Regulatory protein involved in cell cycle and mitosis.	-1.12	1.11
K-Ras	16762399	V-ki-ras2 kirsten rat sarcoma viral oncogene homolog	Signal transduction by p53- negative regulation of apoptotic process-negative regulation of cell differentiation-positive regulation of cell proliferation-positive regulation of cell growth.	-1.05	NC
MSH2	16879883	mutl homolog 2, colon cancer	Cell cycle arrest-Damage response.	-1.1	1.18
DNA Repair					
DDB2	16724471	Damage specific DNA binding	Necessary for the repair of damaged DNA.	1.07	-1.04

BRCA1	16845349	protein 2 Breast cancer 1	Play role in DNA repair of double-stranded breaks	-1.13	1.13
RRM2B	17079808	Ribonucleotide reductase M2 B	Necessary for DNA synthesis-response to DNA damage stimulus.	NC	-1.12
PCNA	16916958	Proliferating cell nuclear antigen	In response to DNA damage, is ubiquitinated and is involved in the RAD6-dependent DNA repair pathway.	-1.11	1.09
Cell Differentiation					
LIN28A	16661155	lin-28 homologA	Negative regulation of glial cell differentiation	1.05	NC
LIN28B	17011255	lin-28 homologB		1.03	NC
EGR1	16989736	early growth response 1	Required for differentiation and myogenic mitogenesis.	-1.01	1.05
MYOD 1	16722526	myogenic differentiation 1	Regulate differentiation	NC	1.04
ZNF675	16870978	zinc finger protein 675	Cancer differentiation	NC	2.04
P53 Signaling					
MDM2	16767332	Mouse double minut2	Promote tumor formation by targeting tumor suppressor proteins, such as p53, for proteasomal degradation.	1.08	-1.07
SESN2	16661544	Sestrin2	Regulate cell growth and survival	NC	1.02
MMP2	16819062	Matrix metallopeptidas2	Positive regulation angiogenesis	1.04	-1.03
GPR87	16960567	G protein-coupled receptor 87	Role in signal transduction	NC	-2.01
ZNF7	17073961	Zinc finger protein 7	Regulation of transcription	NC	-1.01
IGFBP3	17057478	Insulin-like growth factor binding protein 3	Prolonging the half-life of IGFs and altering their interaction with cell surface receptors	1.14	NC
INSR	16867915	Insulin receptor	Cellular response to insulin stimulus	-1.12	1.08
HPRT1	17107045	Hypoxanthine phosphoribosyltransferase 1	Cellular response to insulin stimulus	-1.11	NC
SCARA 3	17067314	Scavenger receptor class A ,member 3	Involved in oxidative stress	NC	1.21

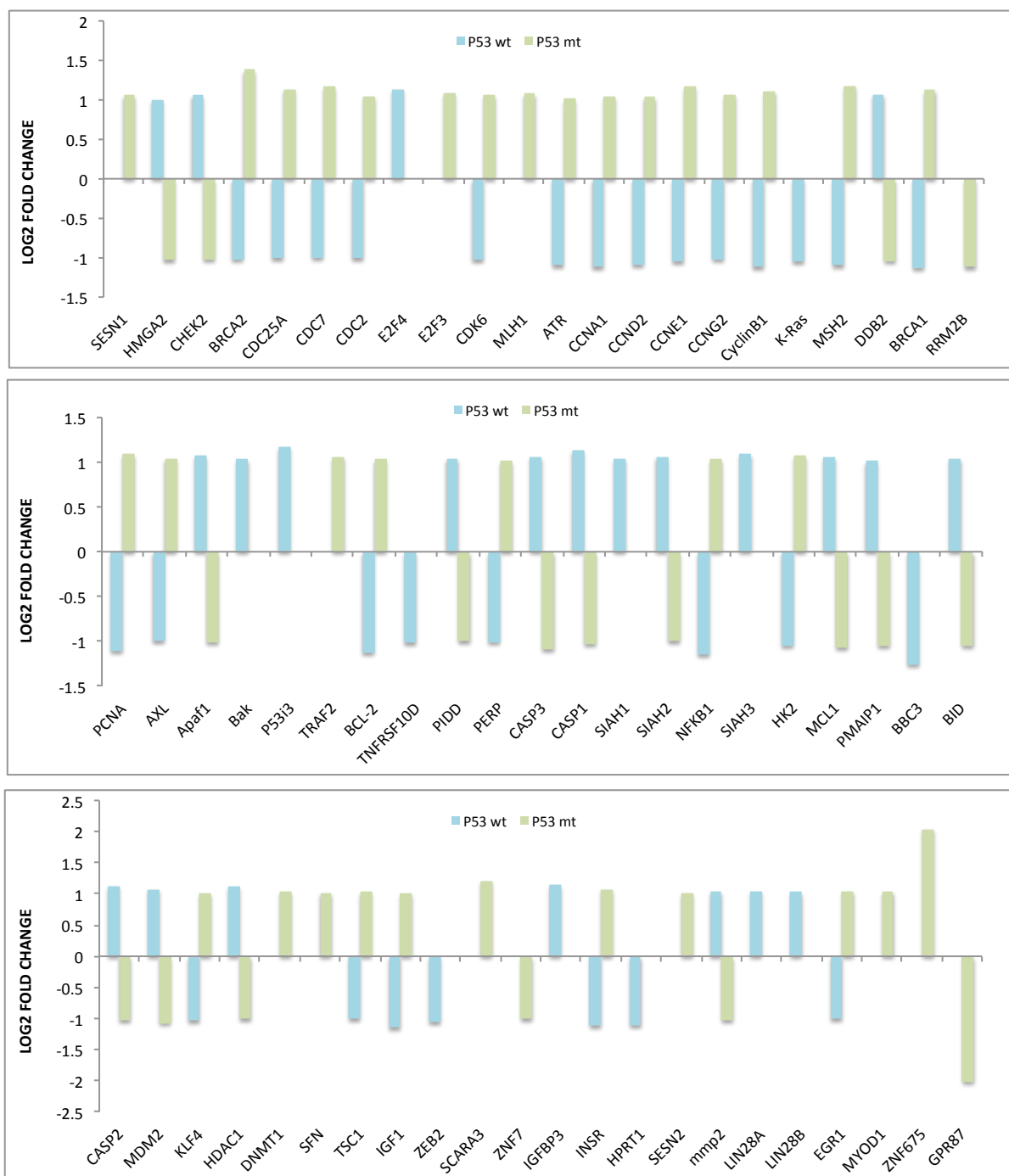


Figure 46: LOG₂ fold change of sixty-five differentially expressed p53 target genes from wtP53-H1299 and mtP53-H1299 transcripts.

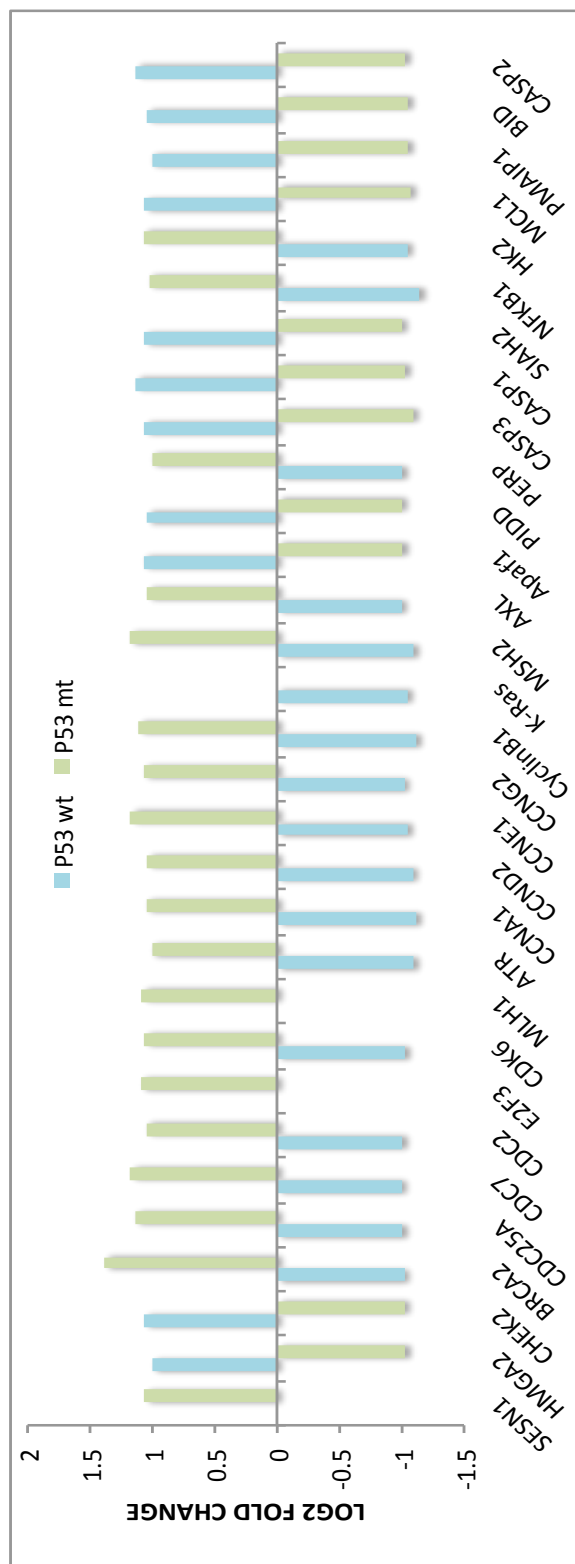


Figure 47: LOG₂ fold change of the most critical differentially expressed target genes in p53 pathway from wtP53-H1299 and mtP53-H1299 transcripts. These genes are involved in cell cycle and apoptosis events

Correlation of p53 Status with Viral growth

After we examined the functional status of p53-R175H mutant, we wanted to know the correlation between the obtained result and the efficiency of Ad5dlE1b55kDa replication. Therefore, we compared the expression profiles of both RNA transcripts that were obtained from the infected samples, which were transfected with wild type and mutant p53 and infected with Ad5dlE1b55kDa, after their normalization with the cellular background (control sample) to identify which p53 responsive genes in the mutant p53 transcript are potential targets for initiating viral replication. These results helped us to understand the rescuing mechanism underlying selective oncolysis of Ad5dlE1b55kDa at the basis of up and down regulation of gene expression.

The results showed that from a total of 232 p53 target genes, 107 were common and shared the same expression patterns in both samples (shown in appendix). These genes have major functions in cell cycle regulation, DNA apoptosis, and cell proliferation. However, forty-five genes were differentially expressed by two fold or more and if these genes were not found in the previously examined mutant p53 transcript for the mock-infected sample, this means that differentially regulated genes in mutant p53 transcript were potential targets for oncolytic Ad5dlE1b55kDa mutant. These genes are involved in cell cycle, apoptosis pathway, proliferation and p53 signaling. Eighty genes demonstrated no change in the expression pattern relative to cellular background. Results are shown in Table 18 and in Figures 48, respectively, as well as Figure 50 that represents volcano plot.

Table 18: P53 regulated genes in response to Ad5dlE1b55kDa in wt/mt p53-transfected H1299 cells. Grouping by functional category of genes whose average expression > or < 2 fold change.

Symbol Caegory	Affymetrix gene ID number	Gene Name	Specific/Other Categories	LOG ₂ fold	Change
				E1bwtp53 /Mock	E1bmtp5 3/Mock
<i>Apoptosis</i>					
FAS	16707149	Fas cell surface death receptor	Apoptosis signaling, p53 Signaling, death receptor signaling, Myc Mediated Apoptosis Signaling.	NC	-1.18
FASLG	16673928	Fas ligand	Interaction of FAS with this ligand is critical in triggering apoptosis	NC	-1.11
ATM	16730845	Ataxia telangiectasia mutated	Cdc25 and chk1 Regulatory Pathway in response to DNA damage, Cell Cycle, p53-signaling pathway.	NC	1.27
Bax	16863922	BCL2-associated X protein	Induction of apoptosis, p53 Signaling, death receptor signaling, Myc Mediated Apoptosis Signaling, Interferon Signaling, promotes apoptosis by binding to and antagonizing the Bcl-2 protein.	NC	-1.01
PIDD	16734119	P53-induced death domain protein	Induce cell apoptosis in response to DNA damage, role as an effector of p53-dependent apoptosis.	1.0	-1.12
TRAF2	17091444	TNF receptor associated factor 2	Inhibitor of apoptotic process.	-1.01	NC
NOXA	16903356	BH3-only member of the Bcl-2 family	Pro-apoptotic protein	NC	-1
PERP	17024187	P53 apoptosis effector related to PMP-22	Apoptosis, Direct transcriptional target of p53, p53 signaling pathway.	-1.16	1.05
CASP8	16889475	Caspase 8, apoptosis-related cysteine peptidase	Caspase Cascade in Apoptosis, D4-GDI Signaling Pathway, FAS signaling pathway, involved in the programmed cell death induced by Fas and various apoptotic stimuli.	1.03	-1.04
CASP1	16743890	Caspase 1, apoptosis-related cysteine peptidase	Positive regulator of apoptotic caspases	NC	1.32
TP53BP2	16699706	Family of p53 inretactin proteins	Regulates apoptosis and cell growth	1.05	1.46

Cyc1	17073565	Cytochrome c	Involved in Mitochondrial Dysfunction and transport	1.03	-1.02
BIRC5	16838359	Baculoviral IAP repeat containing 5	Negative regulation of apoptotic process positive regulation of cell cycle.	-2.4	2.42
ITGB3	16835158	integrin, beta 3	Negative regulation of cell death-positive regulation of angiogenesis	NC	2.01
ESPL1	16751709	Extra spindle pole bodies homolog 1	Apoptotic process, mitotic cell cycle, cytokinesis	NC	-2.05
MAD2L1	16979389	MAD2 mitotic arrest deficient-like 1	Negative regulation of apoptotic process, cell division	NC	-2.21
Apaf1	16755542	apoptotic peptidase activating factor 1	Intrinsic apoptotic signaling pathway	NC	1.01

Cell proliferation and growth

EAF2	16944574	Associated factor 2	Regulation of cell growth-positive regulation of transcription-intrinsic apoptotic signaling pathway.	NC	2.4
ZEB2	17117888	Zinc finger E-box-binding homeobox 2	Negative regulation of transcription, nervous system development, biosynthetic process	NC	1.1
K-Ras	16762399	V-Ki-ras2 Kirsten rat homolog	Regulation of cell proliferation, signal sarcoma viral oncogene transduction by p53.	NC	1.02

Cell cycle

CDKN2A/ARF	17092881	Cyclin-dependent kinase inhibitor 2A (inhibits CDK4)	P53 signaling pathway, Cell Cycle: G1/S Checkpoint Regulation, Non-Small Cell Lung Cancer Signaling, Cyclins and Cell Cycle Regulation	-1.11	NC
MYC	17072669	V-myc myelocytomatosis viral oncogene homolog	Transcription factor of many genes, drive cell proliferation, regulating cell growth, apoptosis, differentiation, Cell Cycle: G1/S Checkpoint Regulation.	1.07	-1.0
CDK20	17095525	Cyclin-dependent kinase 20	Cell cycle, multicellular organismal development, protein amino acid phosphorylation	-1.1	1.01
CENPA	16877956	centromere protein A	Mitotic cell cycle	NC	-2.04
HMGA2	16753641	High-mobility group AT-hook2	Chromatin organization, development, cell cycle, regulation of Cyclin A transcription, cell division, regulation	1.12	NC

CCNB1	16985599	cyclin B1	of growth, mitosis. Response to DNA damage stimulus mitotic cell cycle, spindle checkpoint	NC	-2.19
CENPK	16996722	centromere protein K	Mitotic cell cycle positive regulation of transcription	NC	-2.27
KIF15	16939960	kinesin family member 15	Mitosis	NC	-2.37
ASCL1	16755958	achaete-scute family bHLH transcription factor 1	Positive regulation of cell cycle negative regulation of apoptotic process	NC	1.14
<i>Cell differentiation</i>					
MGP	16761820	matrix Gla protein	Involved in cell differentiation.	NC	2.23
LIN28A	16661155	lin-28 homolog A	Negative regulation of glial cell differentiation.	NC	-1.04
MYOD1	16722526	myogenic differentiation 1	Regulate differentiation.	1.01	-1.05
<i>DNA repair</i>					
RAD51	16799637	RAD51 recombinase	DNA repair/response to DNA damage stimuli	NC	-2.04
H2AFX	16745236	H2A histone family, member X	Positive regulation of DNA repair/ response to DNA damage stimulus	NC	-2.08
GTSE1	16931384	G2 and S phase expressed 1	DNA damage response	NC	-2.14
FANCI	16804559	Fanconi anemia, complementation group I	Response to DNA damage stimulus, DNA repair	NC	-2.21
SESN1	17022362	Sestrin1	Cellular response to DNA damage	NC	-1.11
<i>P53 signaling pathway</i>					
MDM2	16767332	Mouse double Minute 2	An inhibitor of p53 transcriptional activation, ATM, signaling Pathway cell cycle, cell, cycle: G2/M checkpoint, negative effector of Fas and TNF.	1.09	-1.11
Tp53	16840732	Tumor protein p53	Regulates cell cycle by arrest cell growth, activate DNA repair proteins, initiate apoptosis.	3.02	1.4
MDM4	16676343	Mdm4 binding protein homolog	inhibits p53 by binding its transcriptional activation domain.	-1.1	1.08
ZNF10 7	17056470	Zinc finger protein 107	Regulation of transcription.	NC	-2.17

SCARA 3	17067314	Scavenger receptor class A, member 3	Induced by oxidative stress.	-1.28	NC
ESR1	17013809	Estrogen receptor 1	Activation of transcription.	NC	-1.09
ZNF7	17073961	Zinc finger protein 7	Activation of transcription.	1.09	NC
GALE	16683397	UDP- Galactose 4 epimerase	Metabolism	1.02	NC

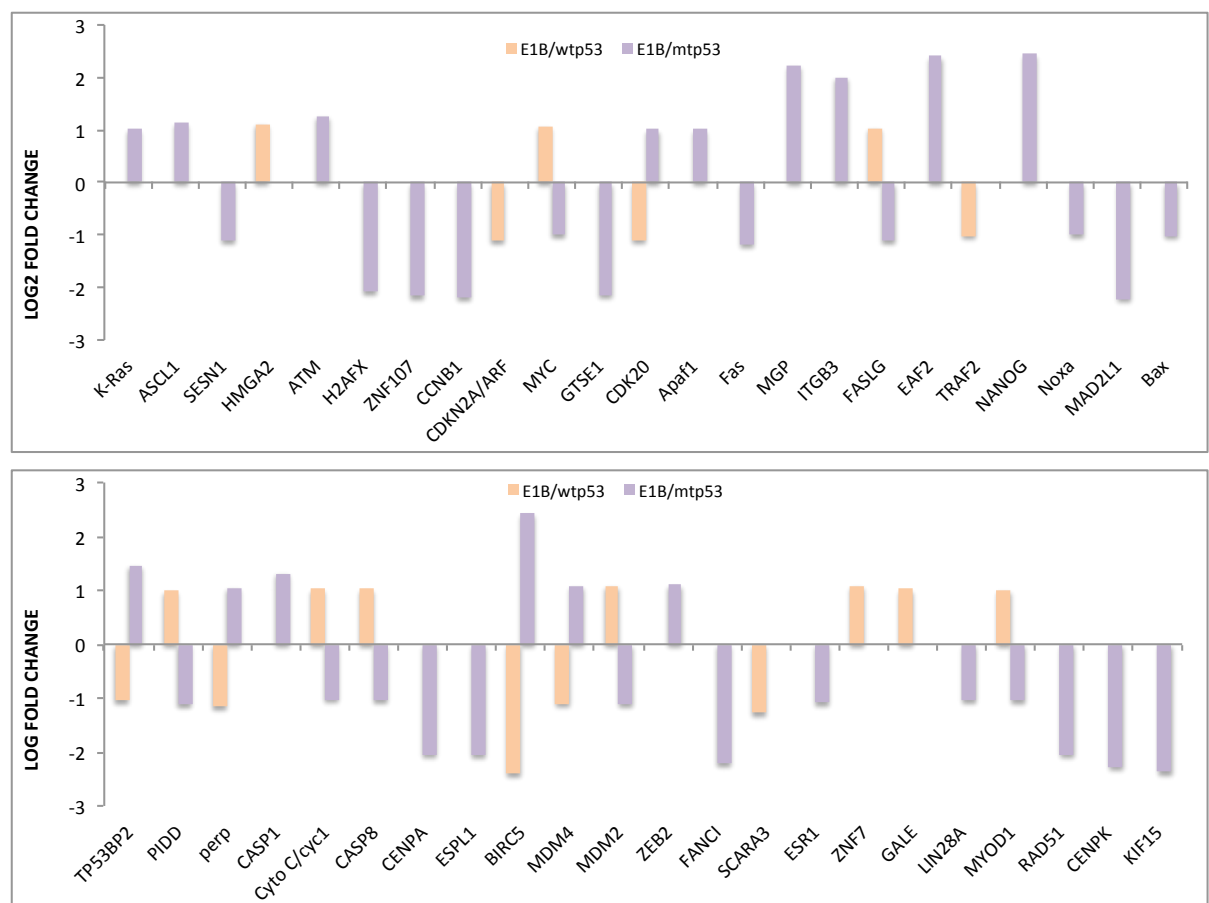


Figure 48: LOG₂ fold change of forty-five differentially expressed p53 target genes from wtP53-H1299 and mtP53-H1299 transcripts infected with Ad5dlE1b55kDa that are critical and have major functions in cell cycle, DNA repair and apoptosis.

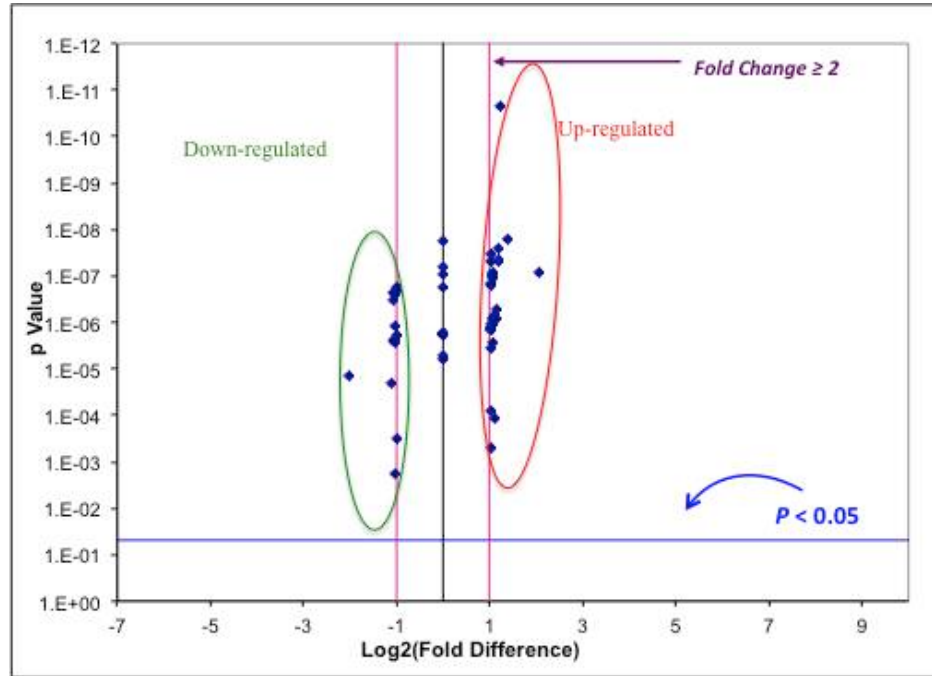


Figure 49: Volcano plot represents the LOG_2 fold ratio of expression level of differentially regulated target genes that are $<$ or $>$ 2 fold change difference in mtp53 transcripts and t-test p-values < 0.05 .

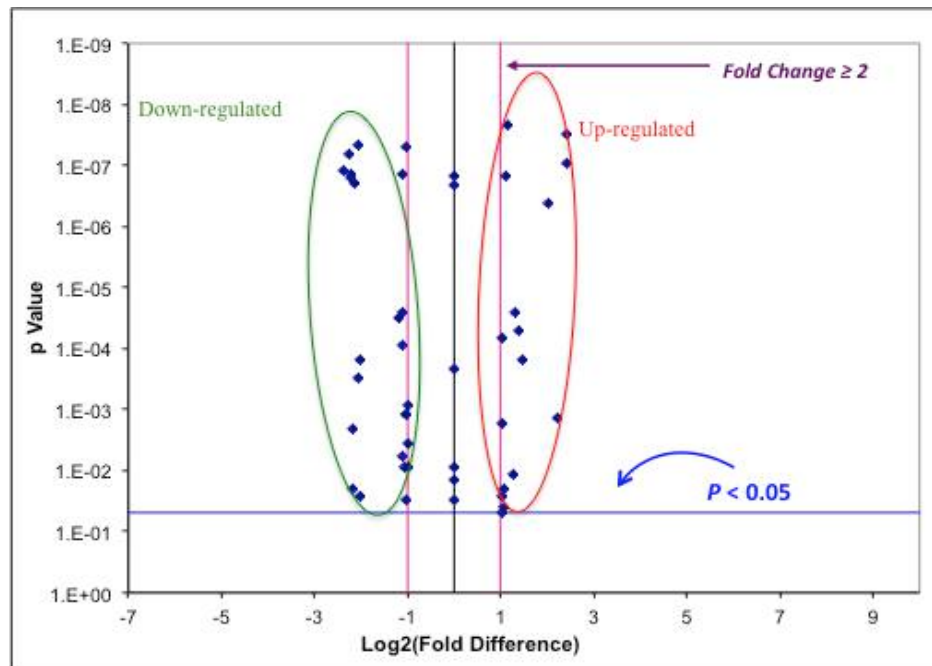


Figure 50: Volcano plot represents the LOG_2 fold ratio of expression level of potential target genes that are $<$ or $>$ 2 fold change difference in response of Ad5dlE1b55kDa replication and t-test p-values < 0.05 .

Validation of putative target genes expression from mock-infected samples and infected samples using RT-qPCR

To confirm the microarray data, twelve RNAs of the putative target genes that showed differential expression were subjected for RT-qPCR in order to evaluate their up or down regulation. Five out of the twelve genes were chosen from the mock-infected comparison based on their involvement in distinct function. The relative expression of each gene was examined by using specific primers for each. The expression level of the identified target genes was measured through the normalization with 5S gene expression. Relative expression between mutant p53 transcript / wild type p53 transcript and control transcript was calculated afterwards as LOG_2 fold change using the equation $\text{LOG}_2 (2^{-\Delta\text{Ct}}_{(\text{target gene})} \text{mutant p53} / 2^{-\Delta\text{Ct}}_{(\text{target gene})} \text{Control})$ and $\text{LOG}_2 (2^{-\Delta\text{Ct}}_{(\text{target gene})} \text{wild type p53} / 2^{-\Delta\text{Ct}}_{(\text{target gene})} \text{Control})$. The relative gene expression data of mock-infected mutant p53 transcript showed three downregulated genes, one upregulated gene and one gene unchanged. However, PCR data for the mock-infected wild type p53 transcript showed three upregulated genes and two downregulated genes. These results, which are listed in Table 19, were in agreement with microarray data with small standard deviation values.

Table 19: LOG_2 fold change in the expression of the 5 target genes of mutant and wild type p53 transcripts relative to control cellular transcript.

(Red – Two fold or more up-regulated, Green – Two fold or more down-regulated, and Black – Minimally Deregulated / Unchanged)

Gene Symbol	Gene Description	(Mutant p53/ Mock control)	(Wild type p53/ Mock control)
CASP2	Caspase 2, apoptosis related cystine peptidase	-1.20	0.90
CHEK2	Checkpoint kinase 2	-1.10	1.00
ATR	Ataxia telangiectasia and Rad3 related	1.90	-0.7
ZEB2	Zinc finger E-box-binding homeobox 2	-	-1.5
MDM2	Mouse double minute 2	-1.19	1.40

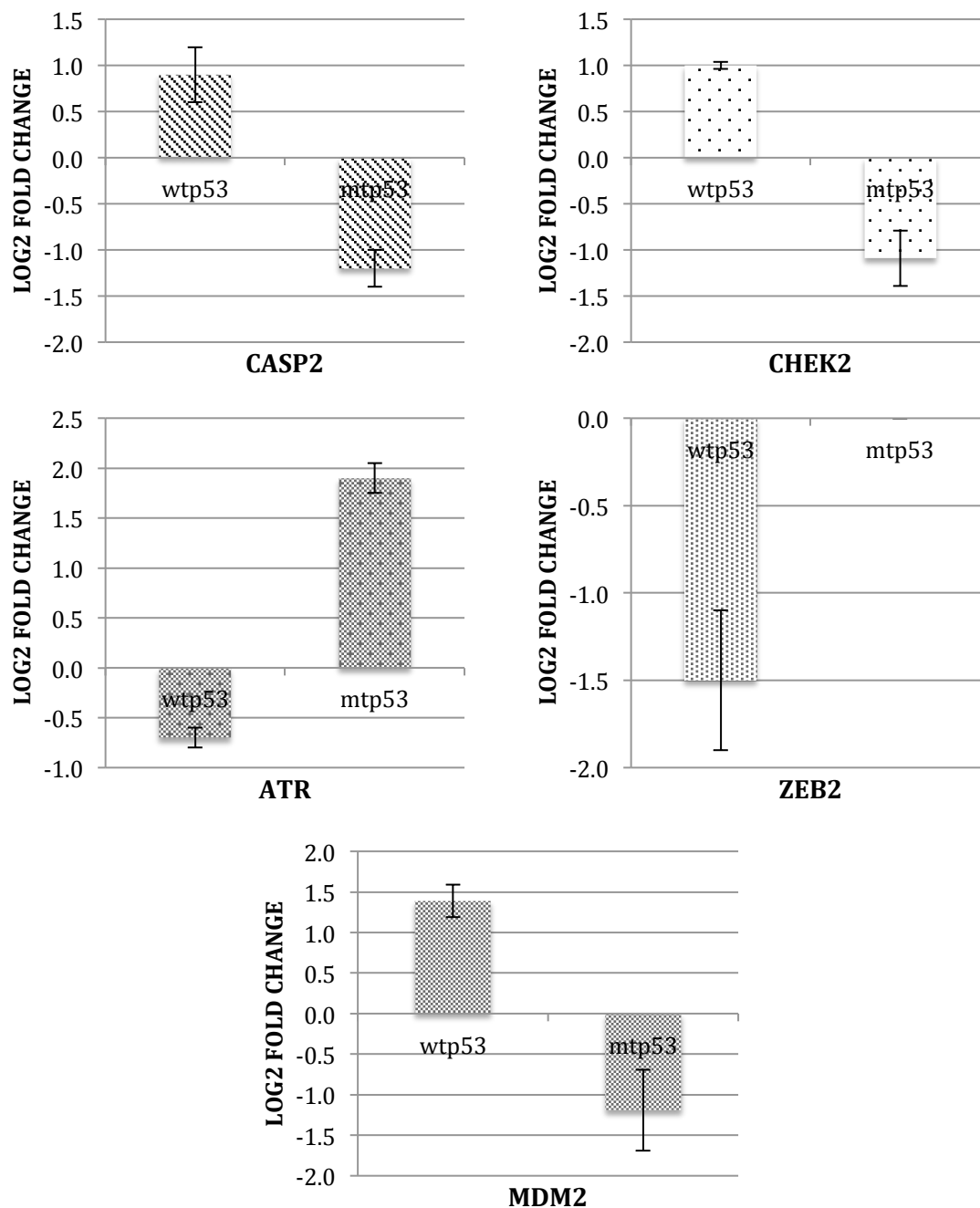


Figure 51: RT-qPCR validation of 5 putative p53 targeted genes relative expression in cells transfected with wild type p53 and mutant p53 transcripts relative to average mock control.

Table 20: LOG₂ fold change in the expression of the 7 target genes of mutant and wild type p53 transcripts relative to control cellular transcript.

(Red – Two fold or more up-regulated, Green – Two fold or more down-regulated, and Black – Minimally Deregulated / Unchanged.

Gene Symbol	Gene Description	(Infected mutant p53/ Mock control)	(Infected wild type p53/ Mock control)
Fas	Fas cell surface death receptor	-0.8	0.1
Bax	BCL2-associated X protein	-1.7	0.3
ATM	Ataxia telangiectasia mutated	1.7	-1.2
ZEB2	Zinc finger E-box-binding homeobox 2	1.7	0.3
MYC	V-myc myelocytomatosis viral oncogene homolog	-	1.6
MDM2	Mouse double minute 2	-1.4	1.3
TP53	Tumor suppressor p53	1.0	2.5

Relative expression of the remaining seven genes between the infected mutant p53 transcript and the infected wild type p53 transcript relative to the control transcript was calculated as LOG₂ fold change using the equation $\text{LOG}_2 (2^{-\Delta\text{Ct}(\text{target gene})} \text{infected mutant p53} / 2^{-\Delta\text{Ct}(\text{target gene})} \text{Control})$ and $\text{LOG}_2 (2^{-\Delta\text{Ct}(\text{target gene})} \text{infected wild type p53} / 2^{-\Delta\text{Ct}(\text{target gene})} \text{Control})$. The relative gene expression data of the infected mutant p53 transcript showed three down regulated genes, three upregulated genes and one gene unchanged. However, PCR data for infected wild type p53 transcript showed three up regulated genes, one downregulated gene and three unchanged genes. These results, which are listed in Table 20 and shown in Figures 52, were in agreement with microarray data with small standard deviation values.

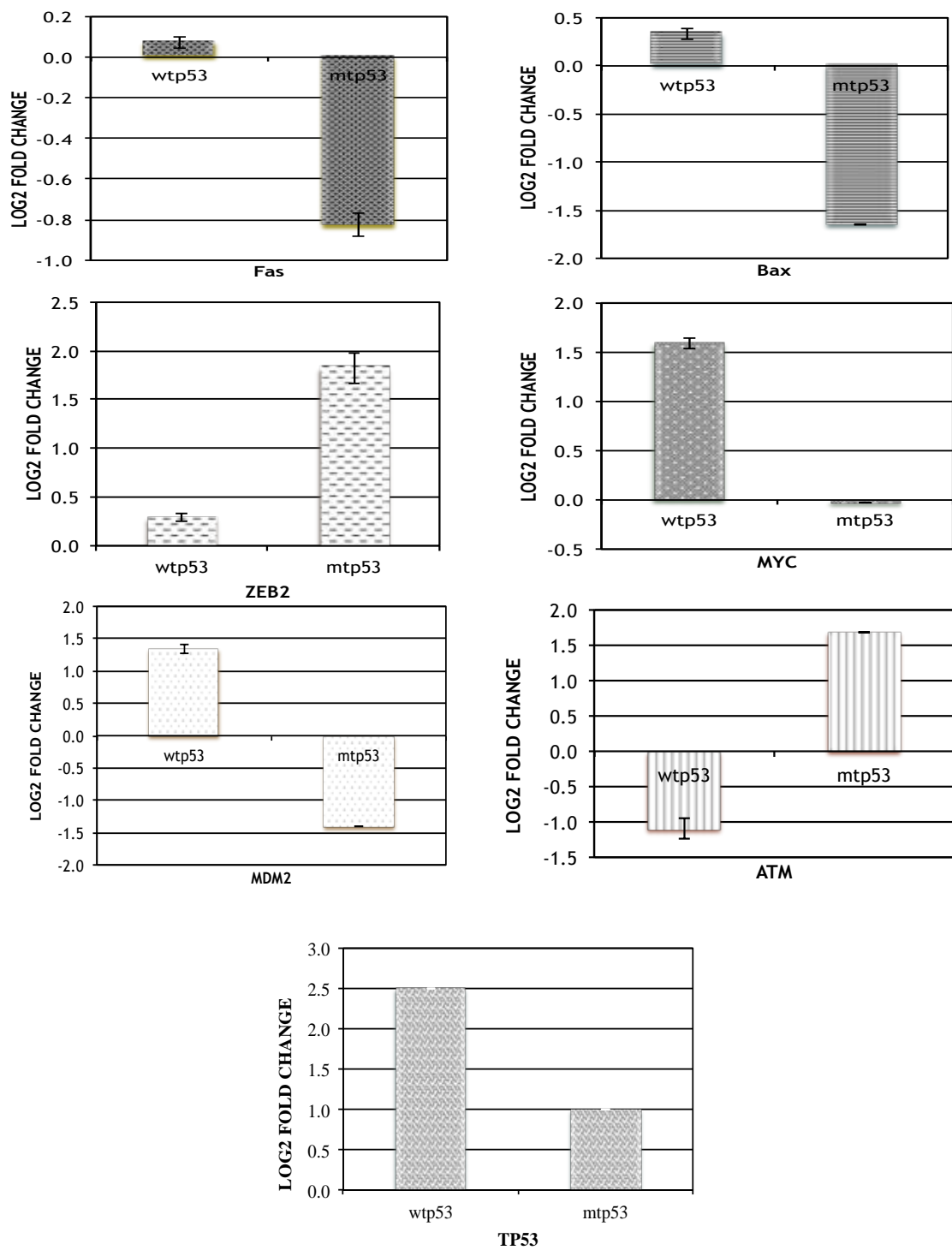


Figure 52: RT-qPCR validation of putative p53 targeted genes' relative expression in Ad5dlE1b55kDa infected wild type P53 and mutant P53 transcripts relative to average mock control.

DISCUSSION

Oncolytic viral therapies have been an innovative approach for cancer therapy in the last century. The ability of selective replication of these viruses has gained them the property of tumor specificity over other cancer therapies. Oncolytic adenovirus was one of the first oncolytic agents that were developed to achieve selective oncolysis, the ideal sort of treatment for cancer. Since the disruption of the p53 tumor suppressor protein is the main target of the viral E1B55kDa oncoprotein and cancer progression, E1B55kDa deleted adenovirus is a promising agent in p53 defective cancers. However, the overall efficacy of oncolytic adenovirus has shown varying degrees of success even when using the same virus. The results have been ambiguous. Therefore, the relationship between p53 genotype status and E1B55kDa deleted adenovirus needs to be studied at the molecular levels. Clearly, increasing our knowledge is a necessity, so that oncolytic adenoviruses can achieve maximum efficiency in terms of clinical efficacy.

The present study was designed to establish a correlation between the replication ability of Ad5dlE1b55kDa and the status of tumor suppressor p53. The strategy was to examine p53 activity after infection at different levels, both quantitatively and qualitatively, and then correlate that with the efficiency of oncolytic viral growth. First, we engineered a p53 transient expression system for examination purposes. Second, we tested the effect of different p53 backgrounds and levels on viral replication efficiency. Lastly, we assessed the functional status of mutant p53 that supports Ad5dlE1b55kDa replication in H1299 cell line and determined the tumor selective viral rescuing mechanism.

P53 transient expression system

P53 mRNA in higher eukaryotes has a half-life as short as few minutes (Rogel *et al.*, 1985). Transgene expression level of p53 in the transformed cells is usually low, and widely variable. Since p53 is a critical key in this study, achieving efficient and long-lived transiently expressed p53 was the objective of this section. Therefore, an expression system was engineered with the purpose of increasing p53 mRNA stability in mammalian cells. Firstly, we tried to increase the chance of vector existence in the cell by improving plasmid DNA stability. Secondly, we tried to elevate the transgene expression level through enhancing posttranscriptional events.

Plasmid DNA stability

The data obtained from plasmid stability in H1299 and HEK293 cells indicates that the presence of different elements within them had different effects on the stability of the plasmids (Figures 15 and 16). Plasmid D, which included the MARs and HPRE elements, remained the most stable with the highest copies per cell at the last points before the plasmid completely lost or degraded. This indicated the positive enhancement of plasmid DNA stability in transfected cells upon the incorporation of MARs and HPRE elements together in the same vector. The effect can be mainly attributed to the HPRE element since plasmids carrying only MARs elements were not as stable as the HPRE-containing plasmid, although MARs are known to facilitate plasmid DNA stability through their binding to nuclear matrix during mitosis (Cockerill and gnrard, 1986; Mirkovitch *et al.*, 1984; Hall *et al.*, 1991; Hall and Spiker, 1994; Jenke *et al.*, 2002). This means that HPRE had a more positive effect on plasmid stability than MARs *in vitro*.

Such effect may be explained by the fact that HPRE is located within the HBV 3' end that contains a common polyadenylation site that prevents plasmid degradation by nucleases. Supporting this explanation is the reduction of the copy numbers of plasmid E that contained a BGH poly-A signal, as it was believed that the homopurine rich sequence in the BGH could make the DNA molecule more susceptible to exo/endo nucleases degradation and structural instability (Ribeiro *et al.*, 2004). If we consider that HPRE is a cis-acting RNA element, then further testing for HPRE role on DNA level is strongly suggested.

On the other hand, in HeLa cells a different picture has emerged. Although plasmid E has shown severe reduction at day 6 post-transfection, which may support the previous conclusion, it exhibited the highest copy number at the last point before the plasmid was completely lost or degraded. However, by considering the standard deviation, it was not significant when compared to plasmids C and D, therefore, it could not be considered as the most stable plasmid in this cell line (Figure 17). Cell type is one parameter for these results, as cellular proteins that may be necessary for regulatory element function may vary between cell types or may be absent, resulting in variation or inhibition of their regulatory function.

Since plasmids can be diluted during mitosis (Pauletti *et al.*, 1990), this would explain why average plasmid copy numbers were reduced after the first harvest point relative to the second harvest point in all transfected cells, as tumor cell lines have an especially high growth rate with fast doubling time, such as 21.8 hours for HeLa cells (Rao and Engelberg, 1966).

P53 expression level

The effect of the regulatory elements was more pronounced on transgene expression than plasmid stability since they can act at the posttranscriptional level to enhance transgene expression *in vitro*. The highest level of gene expression was observed when both MARs and HPRE elements were present in the same plasmid (pD) in the three cell lines. This is consistent with the results of some previous studies on the effect of HPRE on gene expression in HEK293 cells (Xu *et al.*, 2003; Guang and Mertz, 2005; Mähönen *et al.*, 2007).

The previous observation indicates that incorporation of HPRE with the other elements had a greater effect on p53 expression level than without HPRE. This was expected for the following reasons: (a) HPRE is RNA element that mediates RNA nuclear export to the cytoplasm through interaction with cellular factors (Donello *et al.*, 1998), (b) it facilitates and increases RNA accumulation, providing latent expression in the host (Huang and Liang, 1993; Huang and Yen, 1994, 1995) (c) it is also believed that HPRE possesses a polyadenylation site, as it is localized at the HBV 3' end thus enabling RNA stability and 3' end formation and protection from degradation (Sun *et al.*, 2009) , and (d) HPRE may bind to specific cellular proteins to post-transcriptionally regulate gene expression, which can vary depending on the cell type and transgene (Huang *et al.*, 1996; Zang *et al.*, 1998). The latter reason may result in cell or transgene-specific augmentation in the level of transgene expression (Klein *et al.*, 2006), which could explain the differences in p53 expression level that were obtained from the same plasmid (plasmid D) in the different cell lines.

Despite the negative effect of BGH poly-A signal on plasmid DNA stability, it is known to stabilize the transgene mRNA level in the cell through a trade off between plasmid DNA stability and mRNA stability (Ribeiro *et al.*, 2004). However, the results showed that BGH poly-A signal did not have a significant effect on p53 mRNA level when it was compared to the other plasmids in H1299 and HEK293 cells. One assumption could be the presence of a second poly-A signal in the 3' end of the HPRE element that may dispense its function, which likely confirms the previous interpretation of HPRE effect on plasmid stability. Another possible reason is the presence of AU rich sequences in the BGH poly-A signal. As reported in some studies, this sequence may affect mRNA stability depending on the gene and cell type (Caput *et al.*, 1986; Lagnado *et al.*, 1994), which would explain the reduction in p53 expression level that was obtained from plasmid E in both cell lines. On the other hand, in HeLa cells, plasmid E showed the possibility of obtaining the same level of expression as plasmid D at day 12 post-transfection, although it was more stable than plasmid D in terms of plasmid stability, but this stability did not reflect on the transgene expression level. Therefore, the possibility that the BGH poly-A signal may be targeted for degradation is considered.

Interestingly, in H1299 cells and at day13 post-transfection, plasmid C that carries only the two MARs elements displayed a p53 expression level similar to plasmid D although it was not as stable as plasmid D in the previous DNA stability experiment. This confirmed the ability of MAR elements to posttranscriptionally stimulate transgene expression, by a mechanism such as bypass gene silencing (Allen *et al.*, 2000; Levin *et al.*, 2005), more than stabilizing DNA molecules. The same effect on gene expression was also reported in other studies when the transgene was flanked by MARs elements

(Kim *et al.*, 2004; Zahn-Zabal *et al.*, 2001; Girod *et al.*, 2005). Additionally, another *in vitro* study by Cockerill and Grnard in 1986 supported this result, as it reported the presence of ATATAT boxes in MAR element that demonstrated on unwinding property for binding to nuclear matrix and thus augmentation of gene expression (Bode *et al.*, 1992). However, this effect may depend on the cell type, since it did not show the same results in the other cell lines.

To ensure the occurrence of transfection event, all engineered plasmids were co-transfected with β -galactosidase vector (encodes LacZ gene) and transfection efficiency was determined. The results indicate that HeLa cells contain more transfected pDNA than the others, which could be ascribed to many reasons: (a) different cellular uptake ability, (b) more stable plasmids in HeLa cells rather than the others, and (c) different regulatory element effects on plasmid stability. However, since the proportion of transfected plasmids DNA per cell within the same cell type is relatively constant at the first harvest point and also since plasmid D is the most stable among the constructs between the three cell types over time, determining transfection efficiency was not important at this stage.

In conclusion, plasmid D, which contains all elements except BGH poly-A signal, demonstrated the most stability over the others. These elements together have shown not just their post-transcriptional regulation effect on the transgene expression, but they also positively influenced plasmid DNA resistance to nucleases. The positive effect on plasmid-mediated gene expression and stability was mainly obtained from the inclusion of HPRE element in combination with the other elements in the vector. Based on these

conclusions plasmid D was the most suitable choice for long-lived expression, which was needed in this study.

Replication efficiency of oncolytic Ad5dlE1b55kDa in H1299 and HeLa cells

Wild type tumor suppressor p53 protein is a well-known cellular defense against DNA damage and oncogenic activity specifically to viral challenge (Takaoka *et al.*, 2003; Munoz-Fontela *et al.*, 2005). Oncolytic Ad5dlE1b55kDa is able to preferentially replicate in p53-deficient cancer cells with mutant or null p53 status or sometimes in wild type p53 for ambiguous reasons. In this chapter, we tried to evaluate the replication efficiency of Ad5dlE1b55kDa in different p53 cellular backgrounds and levels to find out the mechanism behind the sensitivity of its selectivity.

Since normal cells are not an option for adenovirus oncolysis, H1299 (p53-null) and HeLa (wt-p53) cell lines were chosen as a different source of p53-cancer cells. The results indicated that H1299 cells were more supportive for Ad5wt and Ad5dlE1b55kDaB replication than HeLa cells (Figure 21). Actually, this was interesting since both H1299 and HeLa are considered to be p53 deficient cells. The wild type p53 status of HeLa cells is functionally mutated due to the binding of HPV E6 to p53, causing blockage of its transactivation function. This blockage has been reported as a result of inhibition of p53 acetylation and phosphorylation by HPV E6 region (Thomas and Chiang, 2005; Ajay *et al.*, 2012). However, the high replication rate in H1299 cells was expected because these cells do not contain p53 expression that can hinder their growth.

Unfortunately, this advantage of H1299 cells did not come as a benefit for AdGFP, as this virus still lacked the viral replicative genes ($\Delta E1$) that are responsible for its production, which hindered its replication efficiency. Supporting this explanation, the reduction of its replication rate in HeLa cells as well.

Although Ad5wt and Ad5dlE1b55kDa have a similar replication phenotype, the DNA level of Ad5wt was significantly higher when compared to Ad5dlE1b55kDa in the same H1299 cell line. This indicated that Ad5wt had more capability in replicating its genome and producing virions than Ad5dlE1b55kDa. This statement was supported to the strong CPE that was observed in Ad5wt-infected cells compared to Ad5dlE1b55kDa-infected cells as shown in Figure 22. One consideration could be the effect of E1B55kDa deletion on the viral replication efficiency of Ad5dlE1b55kDa, which is the more likely reason. Another possibility could be the presence of an inhibitory effect that can be overcome by wild type adenovirus, but not mutant adenovirus. If we consider the latter reason, both viruses should replicate at the same level in HeLa cells, but this was not the case, which led us to ignore this possibility.

Interestingly, when viruses replicated in H1299 cells better than in HeLa cells, this meant that different cellular backgrounds, which could be indirectly related to p53, could affect the replication of oncolytic adenovirus differently.

Effect of p53 levels on the replication of Ad5dlE1b55kDa in H1299 cells

In addition to p53 backgrounds, we also evaluated the replication efficiency of Ad5dlE1b55kDa under various wild type p53 levels (the effect of p53 levels) in H1299

and HeLa cell lines. Since each virus differ in terms of the presence or absence of important viral genes such as E1A and E1B, different replication pattern between viruses and different replication starting points were obtained. This observed difference is mainly ascribed to the expression of functional wild type p53 protein that can directly interact with viral genes affecting viral production rate and efficiency.

Ad5wt has the fastest replication and achieved the greatest rate among the others. It started earlier than the others and reached the highest viral DNA level among them at 72 hours post-infection (Figures 23, 24 and 25). This higher efficiency was mainly due to the presence of E1B55kDa gene, whereas the delayed replication of the other mutant was mainly contributed to the absence of this gene that can counter p53 repression activity toward viral replication and spread. This means that Ad5wt had the ability to quickly inactivate and block p53 activity in favor of virus replication. As a result, p53 cannot induce apoptosis in response to viral infection thus facilitating the E1B transformation of the H1299 cell through the aid of the E1A protein for the favorite of virus production.

This is consistent with previously reported binding of E1B55kDa gene to p53 blocking its activity for efficient wild type adenovirus replication (Yew and Berk, 1992; Yew *et al.*, 1994; Graham *et al.*, 1974; Barker and Berk, 1987). Some of these studies have suggested that E1B55kDa has a transcriptional repressor domain that affects p53 activation activity (Yew and Berk, 1992; Yew *et al.*, 1994). Others studies have elicited the binding of E1B55kDa/E4-orf6 complex to p53, which antagonizes p53-mediated transactivation (Dobner *et al.*, 1996; Nevels *et al.*, 1997).

On the other hand, in the mutant viruses that lack E1B55kDa, p53 was not blocked, therefore, both viruses started to replicate in the late stage compared to Ad5wt. However, the replication efficiency of both mutant viruses was different. This variability can be mainly ascribed to the presence of E1A region in one leading to overcome the restriction and augment the replication of Ad5dlE1b55kDa to a level similar to Ad5wt but in lesser extent, which was shown in the case of the negative control whereas it was more restricted in the presence of p53 expression. The latter was not surprising since in the absence of E1B55kDa, p53 usually takes advantage by promoting cellular apoptosis to stop viral spread. In the case of AdGFP, the deletion of the entire E1 region ($\Delta E1$) caused a severe defect in virus replication efficiency leaving the DNA level stagnant with no increase under the presence or absence of p53. These results confirm the fact that p53 expression had a great effect in hindering viral replication of both mutant viruses at various extents but not in wild type adenovirus.

If we consider the hypothesis that different amounts of p53 can affect the viral replication efficiency to various degrees, different viral DNA level should be obtained under variable levels of p53 expression. In spite of this hypothesis, the different p53 levels had an insignificant effect on the growth rate of all viruses as shown in Figures 23, 24 and 25. Both Ad5wt and AdGFP were either replicated or did not replicate, respectively, in the presence or absence of p53, which is the negative control. The reason for these results in both cases was stated above, but the replication rate of each virus was the same under various p53 levels that expressed from different p53-expressing plasmids (A-E). On the other hand, although Ad5dlE1b55kDa replicated more efficiently in p53 level obtained from plasmid B than the others at 48 hours post-infection, this significance

quickly disappeared at 72 hours post-infection and the viral replication rate reached the same level under the various expression levels. This supports the same results from Ad5wt and AdGFP and suggests that the effect of p53 is likely to be more qualitative (depend on function) than quantitative (amount) and that the functional status of p53 could be the critical key behind its activity.

Effect of p53 levels on the replication of Ad5dlE1b55kDa in HeLa cells

It is known that HeLa cells containing the wild type p53 sequence are functionally inhibited due to the presence of the HPV E6 region that directly binds to p53, thereby facilitating its blockage and degradation. The replication phenotype of the three viruses in HeLa cells was different with a lowest replication efficiency of Ad5dlE1b55kDa. This observed difference is mainly relative to the presence of active p53 in either cases presence or absence of replicative viral genes. Both Ad5wt and AdGFP were replicated at high levels which was not surprising for Ad5wt since it possesses a special mechanism that blocks wild type p53 function to promote cell cycle progression and facilitates its replication (Figure 26). However, the replication of AdGFP to this extent was not expected since it lacks the region (Δ E1) that is responsible for the replication process.

One logical reason that explains this phenomenon is the presence of the E1A region in Ad5dlE1b55kDa rather than AdGFP. The E1A gene in some studies was reported for its ability to overcome the blockage and degradation effect of HPV E6 on p53 in HeLa cells by stabilize p53 function through a phosphorylation process, as part of its function in the absence of E1B55kDa (Chiou and White, 1997; Chiou *et al.*, 1994; Lowe and Ruley, 1993). In addition, it implements p53 dependent apoptosis in the

absence of the entire E1B region (Lowe and Ruley, 1993). These facts explain why Ad5dlE1b55kDa could not promote the replication under these conditions and viral DNA levels started to decrease two hours post-infection. Simply, what occurred is the action of E1A on blocking HPV E6 effect and restoring or stabilizing endogenous p53 function has led to obtain functional active p53, which therefore was able to severely hinder the replication of Ad5dlE1b55kDa in the test plasmids as well as the negative control. Supporting this explanation, the better replication of viruses that lack the entire E1 region (AdGFP). Further support for this explanation was the accumulation of mutant p53 in the case of Ad5wt and AdGFP infection, whereas the cellular level of wild type p53 expression in Ad5dlE1b55kDa was maintained under the normal level. This reveals that the effect of HPV E6 was inhibited and p53 status returned to the normal level otherwise p53 expression level would have stayed the same across the viruses.

It is not a surprise that variant levels of p53 expression had no significant effect on the viral replication rate, as was demonstrated previously in H1299 cells. This finding can be applied to both Ad5wt and AdGFP regardless the starting replication time (Figures 26 and 27). However, if we consider the above stated explanation on Ad5dlE1b55kDa replication, this evaluation is not essential since the viral replication level reached zero in all cases (Figure 28). This agreement in the results on the p53 variant levels has led us to same conclusion in which the oncolytic Ad5dlE1b55kDDa replication efficiency may be based on the activity of p53 not the quantity, which needs an assessment.

Comparison of Ad5wt, AdGFP and Ad5dlE1b55kDa growth in H1299 and HeLa cells

The conclusions that have been reported previously in this study have pushed us to make a relative comparison between the viruses' replication phenotypes in both cell lines. Up until now, it was not clear whether oncolytic Ad5dlE1b55kDa mutant replication efficiency is based on the cell's phenotype or p53 expression but it seems that both are correlated to each other. From the study's results, it looks like every virus had a different replication rate in each cell line, especially Ad5dlE1b55kDa. The reason for this variable selectivity turned out to be the status of cellular p53 whether it is functional active or inactive due to a specific reason, even if it has a wild type sequence.

Ad5wt showed its efficient replication capacity in either cell line because both are p53 deficient, and even the presence or absence of p53 is not important to implement replication in this case (Figure 21). When we compared the virus replication in HeLa cellular background and in PUC19 transfected HeLa, Ad5wt in HeLa background still showed deficiency in replication compared to PUC19 transfected HeLa although both are considered as negative controls. One possibility could be ascribed to the status of the cell cycle at the time of infection, as it was reported earlier that HeLa cells are more readily infected when they are in S phase and because of their rapid cycling, a high percentage of cells are infected (Goodrum and Ornelles, 1997).

Supporting this explanation was the deficiency of AdGFP replication in both cell line backgrounds compared to PUC19 transfected matched cell lines (Figures 21 and 30).

Despite that, AdGFP was found to replicate more efficiently in HeLa cells than H1299 cells. In line with this, the replication of Ad5dlE1b55kDa in defective p53 cells (H1299) to a higher extent in the absence of exogenous p53 expression, as shown in the negative control as well as in the rest of cases (A-E) at 72 hours post-infection, whereas it did not replicate in active wild type endogenous p53 (HeLa) as a result of E1A effect on p53 effect (Figure 31). This comes in agreement with several reports that have also demonstrated the same observation on E1b deleted adenovirus restriction of replication in cancers with wild type p53, but better replication in H1299 cells (Bischoff *et al.*, 1996; Harada and Berk, 1999). In addition, the same trend of the viral DNA replication were obtained after the infection of cellular backgrounds of H1299 and HeLa with the same virus regardless the deficiency of DNA levels which could be due to the previously stated reason of a cell cycle restriction condition (Figure 21).

In conclusion, the replication efficiency of the oncolytic Ad5dlE1b55kDa mutant is more likely to be contributed to the functional status of cellular p53, either active or inactive, or to other cellular factors that are dependent on p53 more than the cellular phenotype or the quantity of p53 expression. This confirms the importance of the examination of functional status of mutant p53 in different cancers to obtain a clearer understanding of the relationship between oncolytic Ad5dlE1b55kDa and p53 status, which was already assessed and discussed in this study in the next chapter, thus a clear vision would then exist for further investigations in the field of oncolytic virotherapy.

The impact of p53 functional status on Ad5dlE1b55kDa growth

Accumulation of genomic alteration in a given cell leads to deregulation of key signaling pathways that control the cell proliferation and fate. Tumor suppressor p53, which is the key cellular defense against unscheduled cell proliferation, is the most targeted gene in genetic alteration as well as in viral infection. Therefore, the mutant form of p53 provides a favorable environment for efficient viral replication. In this study, a genetic means was used to abolish p53's DNA sequence binding ability through the introduction of point mutations in p53 that often affect its functional activity as tumor suppressor, resulting in uncontrolled cell proliferation and viral replication. However, not all mutations are successfully rescued the viral growth in the context of the oncolytic Ad5dlE1b55kDa mutant leading to a deferentially replication phenotype in different p53-deficient cancers. Therefore, in this part of the study, we first assessed the functional activity of mutant p53 that rescued viral growth, and second we determined the impact of this activity on the sensitivity of oncolytic adenovirus replication. In other words, we investigated whether Ad5dlE1b55kDa growth is dependent on p53 function or not.

General observation from the results in Figure 44 showed that Ad5wt replicated more efficiently with one log fold difference than Ad5dlE1b55kDa in all cases, wild type p53, mutant p53 and controls. This was expected and it reconfirms that the E1B55kDa region is an essential requirement in achieving efficient viral replication. This observation is consistent with the previously obtained results in chapter II and with other studies (Kim *et al.*, 2002; Hann and Balmain, 2003). It was not surprising that the obtained DNA level of Ad5wt was the same across the following conditions: cells expressing wild type p53,

mutant p53P72R, mutant p53R175H and the controls. This is because adenovirus wild type is capable of controlling the cell towards its replication using its viral proteins.

On the other hand, Ad5dlE1b55kDa was shown to have the highest DNA level in the expression of mutant p53R175H. This means that oncolytic Ad5dlE1b55kDa replicated more efficiently in this mutant than the other mutants and the wild type p53 as well. This demonstrates that mutant p53R175H rescued viral growth more efficiently than the others, although it did not reached the level of Ad5wt due to the absence of the E1B55kDa region.

Actually, it was not surprising that the expression of wild type p53 restricted viral growth, as wild type p53-expressing cells usually undergo apoptosis in response to viral infection to prevent its spread as a result of p53 activity. Also, the reaction of mutant p53P72R and mutant p53N268D in restricting viral growth was not efficient. The mutations at these positions seem not to affect the protein functionality suggesting that p53P72R and p53N268D mutants were still retaining the wild type function although they are located distantly on the gene. This explanation aids the notion that mutations have a diverse effect on p53 functional activity (De Vries *et al.*, 2002; Dehner *et al.*, 2005; Ang *et al.*, 2006; Joerger *et al.*, 2006) confirming the above suggestion.

If we consider the above conclusion, mutant p53R175H should disrupt p53 functional activity to a high extent, which means that this protein should be functionally inactive, in order to support the replication of Ad5dlE1b55kDa. However, this did not explain the reason for the relative low replication activity of the virus in the null cells (mock-transfected H1299). This suggests either of two possibilities: a) oncolytic

Ad5dlE1b55kDa replication does not correlate to p53 status, but rather to other factors in the cells. If we consider this possibility, we should have seen the same results across all of the conditions as we used the same cellular background, but this was not the case especially when wild type p53 restricted viral growth the most among the other conditions. Or b) mutant p53R175H possess an oncogenic activity or gain of new functions that support the replication of Ad5dlE1b55kDa, which is not provided by the other mutants. In order to confirm this possibility, we assessed and determined the functional status of p53R175H, which rescued the oncolytic viral growth, as well as the gain of function activity.

Functional status of mutant p53R175H that rescued oncolytic Ad5dlE1b55kDa growth

p53 acts as transcriptional activator of a large set of downstream target genes in its pathway that mediate its function including cell cycle arrest, DNA repair and apoptosis (Kern *et al.*, 1991; Farmer *et al.*, 1992; Zambetti *et al.*, 1992). Therefore, we assessed the functional activity of mutant p53R175H along with p53 wild type, as a control, by looking at the expression of downstream target genes at the transcriptional level. The compared results revealed that the expression of wild type p53 and mutant p53R175H led to up and down regulation of many common genes that are known to mediate the function of p53.

Most of the commonly expressed genes are activators and main regulators of downstream genes, which mediate the direct function of cell cycle control and division, or direct inducers of apoptosis and DNA repair. This indicates that these genes are critical

for the cell and are constantly expressed regardless of the functional status of the p53 protein. These results are consistent with other studies that reported the same conclusion with different genes (Mu *et al.*, 2009; Forte *et al.*, 2013).

However, the sixty-five differentially expressed genes were involved in different p53-related biological functions. Generally, most of the genes are more pronounced in apoptosis and cell cycle events. The differentially regulated genes that are required for p53-mediated cell death such as PIDD, CASP3 and BID were upregulated in the wild type p53 transcript whereas in the mutant transcript, these genes showed down regulation by 2 fold and greater. However, the apoptosis effectors and negative regulators including AXL and NFKB1 showed the opposite expression pattern. This means that upon p53 expression in H1299 lung cancer, wild type p53 restored its function in the cell and started to induce apoptosis to stop the proliferation of cancer cells, which is also confirmed by the over expression of antiproliferative genes in the same cell. However, the mutant p53 did not have the ability to induce apoptosis upon expression, which is mainly ascribed to the presence of mutation at 175 hot spot that hinder the ability of p53 to induce apoptosis. Further confirmation of this explanation is the up regulation of proliferative genes in the same cellular transcript including DNMT1 and SFN.

Based on our findings, wild type p53 has restored its function and mutant p53 has lost its functional ability, this is also supported by the fact that the major genes such as cell cycle genes have arrested the cell cycle upon the expression of wild type p53. This was shown in the up regulation of cell cycle key regulators control and down regulation of negative regulators in the same transcript. However, the opposite results were obtained

in the mutant transcript, as the cell cycle was in the progression mode and the key regulators genes were downregulated and would not be able to control the cell whereas the negative regulators such as ATR went up in the same transcript. Consistent with this conclusion is the failure of the mutant p53 in implementing DNA repair event (Table 17).

From the above results of differentially expressed genes, it seems that mutant p53R175H has completely lost its tumor suppressor function. However, if we consider the commonly expressed genes in both wild type and mutant p53 transcripts, some apoptosis related genes were still under control in both conditions and has the same expression pattern. This result has changed the obtained conclusion of complete loss of function suggesting that a partial loss of mutant p53R175H tumor suppressor function is highly likely to be as a result of defective apoptotic control and this loss might be sufficient for the oncolytic virus towards viral replication. This finding also indicates that the impact of mutations on p53 function may vary from a wild type-like activity to a partial function or complete loss of function.

Correlation of p53 status with viral growth

After the assessment of the functional status of mutant p53R175H, it was important to determine whether this partial loss of p53 function was the reason behind the activity of Ad5dlE1b55kDa replication or if this mutation resulted in the acquisition of a new phenotype that caused the successful rescue of the virus. So far, there is no study that investigated the Ad5dlE1b55kDa transcriptional reprogramming in mutant p53R175H as a means to identify deregulated targets and pathways. Both infected transcripts were normalized with the control sample (H1299 RNA) to identify whether the previous

differentially regulated p53 target genes that were found in the mock-infected mutant p53 transcript are the same potential targets for viral replication or if there are different genes that have become the target and correlated with Ad5dlE1b55kDa growth.

Microarray gene expression results of the tested transcripts revealed a 107 commonly expressed genes in both transcripts, which means that both transcripts share some similarities in the context of p53 biological functions. These genes are mostly the consistently expressed genes regardless of any stress. However, the forty-five differentially expressed genes revealed that some genes were upregulated in one transcript but downregulated in the other transcript and vice versa. These genes mediate direct functions in the p53 pathway including apoptosis, cell division, cell proliferation, DNA damage response, mitotic cell cycle and cell growth (Table 18 and Figure 48).

From the obtained results, it seems that the differentially expressed genes are altered during viral infection as a direct effect of the virus. The alteration was more pronounced in the mutant p53 transcript than in the wild type p53 transcript in response to viral replication. This actually was expected since wild type p53 is functionally active and could therefore hinder the virus growth, whereas the mutant p53 has altered activity and cannot inhibit viral growth, resulting in successful replication.

This was evident by two observations: first, the differentially expressed genes between the infected mutant p53 transcript and the infected wild type p53 transcript which revealed that mutant p53 did not have the ability to prevent the cell transformation process triggered by the virus. However, in the case of wild type p53, the virus failed in transforming the cell to the favorable viral growth environment. Of note, the completion

of cellular transformation can be reached by deregulating the expression of proliferative and cell cycle genes towards cell cycle progression. The positive regulators of these genes were upregulated in the case of mutant p53 whereas they were downregulated in the wild type p53 transcript such as K-ras and CDK20 genes. In parallel with this, the negative regulators were shown to have the opposite expression patterns, which confirms the defect of mutant p53 transcript in preventing and hindering the viral replication process resulting in viral growth.

Second, the differentially expressed genes in the infected mutant p53 transcript did not show the same pattern of expression in the mock-infected mutant p53. Genes that are tightly correlated with viral replication were deregulated and showed different expression pattern such as Bax, MYC and Apaf1. This meant that mutant p53 transcript was still not the favorable transcript for Ad5dlE1b55kDa growth although it lost its defense ability towards viral challenge and stress. Therefore, once the virus entered the cell, it changed the expression patterns of these genes in favor of its replication. Supporting this explanation is the deregulation of some genes in the infected mutant p53 transcript that are not found in the mock-infected mtp53 like ZEB2 and NOXA. These genes, which are targets for Ad5dlE1b55kDa, account for viral rescuing activity, which can be considered as gain of new function in this mutant.

Finally, these results together suggested two findings. First, mutant p53R175H transcript is defective in blocking and preventing Ad5dlE1b55kDa growth. Therefore, it was a suitable target for the virus in order to replicate and generate virions even if p53 still retained partial apoptotic function, which could be easily controlled by the viral

proteins. Second, the deficiency in the function of mutant p53R175H transcript indirectly benefits viral replication by allowing the virus to successfully reprogram the expression of some genes in the transcript that are potential targets for Ad5dlE1b55kDa, revealing a mechanism of Ad5dlE1b55kDa selectivity in the context of oncolysis. This indicates that rescuing viral growth is not equivalent to simply losing wild type p53 function, and the allowance of viral growth is an acquired or gained of new function by mutant p53R175H. This conclusion was found to be congruous with another finding, which showed that conformational mutations are more oncogenic than other mutations like DNA contact mutations (Gualberto, *et al.*, 1998).

The obtained findings led us to assume the viral rescuing mechanism at the basis of up regulation or down regulation of gene expression, under the mutant p53R175H properties, which begins with the entrance of Ad5dlE1b55kDa into a given cell. Briefly, once the virus reached the nucleus, it starts to govern the cell cycle, which represented in cell proliferation, cell division, cell growth and mitotic cell cycle. From the previously reported functions of viral proteins in the literature (described in introduction), it seems that the virus directed its proteins to deregulate the expression of many genes in the network to control the cell cycle, creating an optimal environment that supports its replication.

Early E1A coupled with E1B19kDa work together in transforming the cell to S phase, the DNA synthesis phase, which is a required event for viral replication (Ruley, 1983). Firstly, E1A interfered with cellular genes that are known to activate the proliferative and growth genes, which was demonstrated by the induction of EAF2, ZEB2

and K-ras genes. E1A also regulated the expression of cell cycle players by down regulating cyclin kinase inhibitors such as CDKN2A, up regulating cyclin dependent kinases like CDK20 and delaying the mitotic cell cycle process for long-lived S phase in favor of synthesizing more viral DNA. These results were in agreement with the known function of E1A viral protein in down regulating negative regulator of cell cycle control (Dyson and Harlow, 1992).

The deregulation of the cell cycle by E1A activates the cellular mechanism to implement p53 dependent apoptosis, which is inhibited by E1B19kDa to prevent the premature death of host cells during productive infection (White *et al.*, 1992). The latter is not just blocking p53-dependent apoptosis, extrinsic pathway, but also p53 independent apoptosis, intrinsic pathway (Sabbatini *et al.*, 1997) in a proper way for efficient productive viral replication. This fact was demonstrated in our study as many apoptosis-related genes showed down regulation in their expression as a result of E1B19kDa targeting to prevent the premature cell death, facilitating viral DNA synthesis and production.

These genes are divided between the extrinsic (cell death receptors signaling) and intrinsic (mitochondrial signaling) related genes, in which the extrinsic genes started with the FAS death receptor and FASLG and continued down an apoptotic cascade passing key molecular components including BID and CASP8, critical activators of intrinsic genes pathway. Since FAS and FASLG were downregulated, all the downstream dependent genes showed down regulation in their expression including BID and CASP8.

CASP8 also cleaves and activates a downstream caspase cascade that implements apoptosis (Martin *et al.*, 1998; Lin *et al.*, 1999), which did not occur here as a result of its down regulation. Therefore, the downstream caspases did not show any activation as their expressions were down (shown in Table 18), and they eventually failed to initiate apoptosis. This suggests that E1B19kDa succeeded in blocking the cell from undergoing premature apoptosis, which is also an important activity for adenovirus to evade immune surveillance of the host. Supporting this conclusion is the downregulated expression of pro-apoptotic genes such as NOXA and Bax, a direct transcriptional target of p53 (Miyashita and Reed, 1995).

Also, E1A targeted different genes that are known to affect virus goal (growth) such as MYC, which function as a key cellular regulator of many genes, therefore it was not surprising when it serves as a major target for down regulation by viral proteins. This was previously reported as part of the virus strategy to create an optimal cellular environment for its replication (Ben-Israel *et al.*, 2008). In addition, MDM2 was targeted for down regulation by the virus since it possesses an anti-viral response thus inhibiting its interaction with p53, which could be already inhibited due to a mutated form of p53. The two ways inhibition prevented p53 degradation by MDM2 leading to its accumulation in the cell. This was seen in the down regulation of MDM2 expression by greater than two fold, and p53 also went up by two fold and greater resulting in its stabilization. This result is consistent with the fact that MDM2 is a negative regulator of p53 and this stabilization is due to a failed interaction between both genes (Buschmann *et al.*, 2000). Further evidence of viral targeting was demonstrated in another study when MDM2 expression was rapidly regulated during E1B deleted adenovirus infection

resulting in enhanced viral gene expression (Yang *et al.*, 2012). A brief illustration that shows the assumed viral rescuing mechanism is shown in Figure 53.

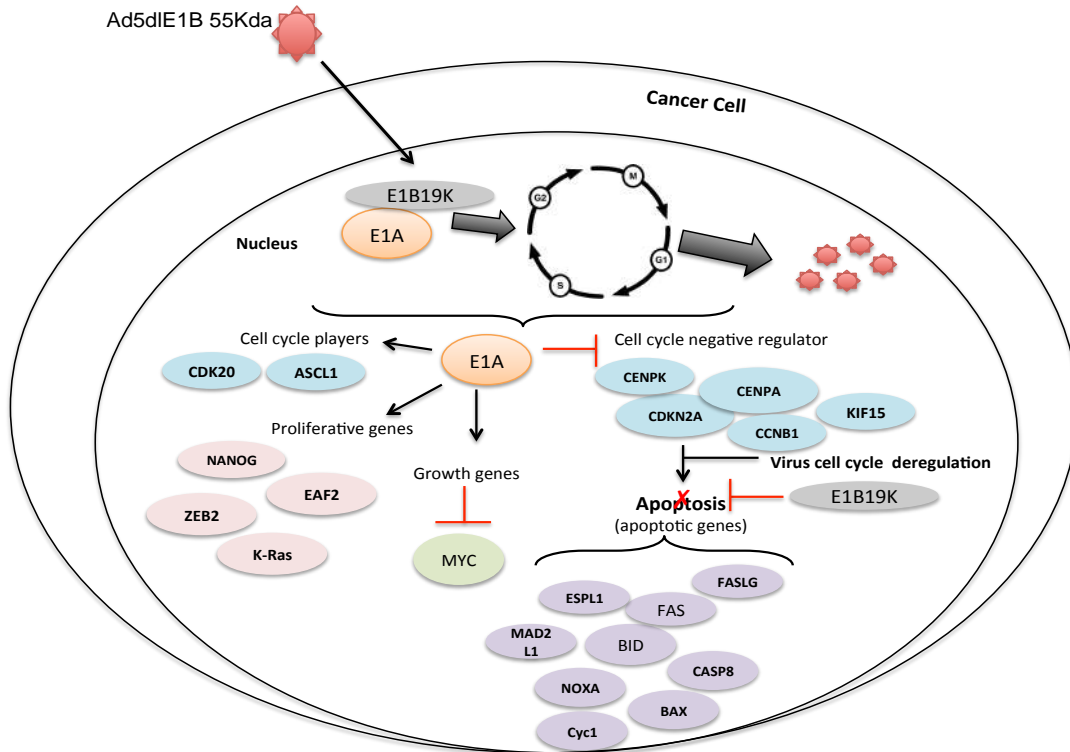


Figure 53: An illustration of E1B55kDa-deleted adenovirus rescuing mechanism.

Taken together, it seems that successful Ad5dlE1b55kDa replication entailed deregulation of cell cycle genes towards cell progression, which was achieved as a result of dysfunctional cell cycle control. It also entailed elimination or inhibition of premature cell death and bypassing of host antiviral mechanisms. This result does not mean that reported genes in this study are the only genes that are targeted by Ad5dlE1b55kDa, but since they are out of our study's scope, another study on these genes is suggested for further understanding. However, logically those reported genes that were found in this study are the main targets for viral growth since they control apoptosis and the DNA synthesis phase, which are the main steps towards viral production.

CONCLUSION

In this project the efficacy of oncolytic Ad5dlE1b55kDa mutant in correlation with the status of p53 was studied by firstly evaluating Ad5dlE1b55kDa growth in different p53 backgrounds, levels and functional activity, and secondly by investigating the effect of mutant p53 on viral growth ability. Based on the presented data in this thesis, we can conclude the following:

- 1- Post-transcriptional regulatory elements have a positive effect on transgene expression and plasmid DNA stability but at various degrees. Among the tested elements in this study, the inclusion of hepatitis posttranscriptional regulatory element (HPRE) in the expression vectors has positively influenced plasmid DNA stability as well as gene expression through exerting the following functions: RNA export and accumulation, increase mRNA stability and posttranscriptionally regulate gene expression. The effect on plasmid DNA stability needs to be further investigated.
- 2- The replication efficiency of oncolytic Ad5dlE1b55kDa is correlated with cellular p53 activity, but not with the level and the quantity of wild type p53 expression.
- 3- The differential replication ability of oncolytic Ad5dlE1b55kDa is dependent on the functional status of mutant p53. Mutant p53R175H is a supportive mutation as it supported viral growth by exerting two distinct activities: loss of function and gain of function. Loss of function by losing the transcriptional activity of target genes that mediate defense mechanism against viral challenge whereas gain of

function is by allowing the deregulation of transcription of genes that facilitates viral growth.

- 4- The successful Ad5dlE1b55kDa replication entailed cell transformation to the progression mode, inhibition of premature cell death and bypass host antiviral mechanism through targeting and deregulating cell cycle genes, apoptosis related genes and antiviral genes.

In summary, our study revealed that the replication ability of oncolytic Ad5dlE1b55kDa seems to be dependent on p53's functional activity in terms of loss and gain of function activity. Therefore, it will be necessary to assay all mutations that support Ad5dlE1b55kDa growth for their functional activities to determine how these mutations are rescuing viral growth and whether these mutations share the same transcripts (mRNA profile) or not. This finding was based on an unbiased correlation between the p53 status and the replication ability of oncolytic Ad5dlE1b55kDa. Therefore, in the context of oncolytic virotherapy, examining p53 status will have a prognostic significance. Furthermore, knowledge about the functional status of p53 would potentially help in determining the therapeutic outcome and would have a major impact on patients' survival.

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APPENDIX

List of commonly expressed genes in the four tested samples (wtp53, mtp53, E1b/wtp53 and E1b/mtp53) after normalization with H1299 cellular RNA.

SEMA3F	CCNE2	PTEN	VEGFA
BAI1	CyclinD3	EGFR	IGF1R
CDC6	GADD45A	ZEB1	GDF15
BTG2	GADD45B	NANOG	IGFBP6
CHEK1	p53BP1	SOX2	FADD
TSC2	XPC	CD44	FOXO3
CDC25C	Bcl-6	NOTCH	KAT2B
CDK9	p53AIP1	SIRT1	PRC1
CDK8	CASP6	LDHA	PRKCA
RPRM	GML	ASCL4	XRCC5
ATF3	Tp53	XRCC5	

List of commonly expressed genes in the mock-infected samples (wtp53 and mtp53) after normalization with H1299 cellular RNA.

SEMA3F	CCNE2	CDK1	Fas	WIP-1/P21	ASCL1	TNFRSF10B	KAT2B
BAI1	CyclinD3	CDK2	ATM	PTEN	ASCL4	IGFBP6	PRC1
CDC6	GADD45A	CDK4	Bax	EGFR	VEGFA	GALE	PRKCA
BTG2	GADD45B	CDK20	TP53BP2	ZEB1	IGF1R	ATF3	RPRM
CHEK1	p53BP1	CCNB2	CASP8	NANOG	IRS2	BIRC5	STAT1
TSC2	XPC	CyclinD1	CASP6	SOX2	Tp53	FADD	TADA3
CDC25C	CDKN1A	cyclin B3	CASP9	CD44	Pin1	FASLG	XRCC5
CDK9	CDKN2A	GADD45G	cyc1	NOTCH	GDF15	FOXO3	B2M
CDK8	E2F2	Bcl-6	CASP8	SIRT1	GTSE1	JUN	TNFRSF6
MYC	E2F1	PTTG1	GML	LDHA	TP53INP1	MDM4	p53AIP1
KAI	TP73						

List of minimal change (NC) expressed genes in the mock-infected samples (wtp53 and mtp53) after normalization with H1299 cellular RNA.

GD-AIF	P48	RPS27L	Vascular smooth muscle alpha-actin
PAI	CHK1	PAG608	Collagen type XVIII alpha 1
Maspin	P53R2	EF-1a	Neurofilament subunit NF-L
TSP1	Noxa	Zac-1	NB Thymosin beta
TP63	P53DINP1	E124/PIG8	LIM domain protein
P21 WAF1	KILLER/DR5	P2XM	cAMP activated protein kinase B
Snoothelin	CSR	MCG10	Lysosomal Mannosidase alpha B
GOS2	PIG3	TauT	TGF-Beta Superfamily protein
14-3-3s	PIR121	SCOTIN	Multiple exostoses type II protein
VLDL	PUMA	p85	purine rich element binding protein A
PTPA	PA26	IL2	basic transcription factor 2 p44
c-HA-ras 1	CART	CENPA	Histone 2A-like protein
DM Kinase	Fra-2	ESPL1	p14ARF
DRAL-FHL2	ESR1	H2AFX	PTG2
ZNF127-Xp	PCBP4	ZNF107	Wip1
LISCH7	RB1	CCNB1	IL6
Tip-1	RELA	FANCI	cMet
NF-116	EAF2	MAD2L1	ABC3
MIHB	MGP	CENPK	Quiescin/QSCN6
MIHC	ITGB3	KIF15	Adipophilin
IFNB1	RAD51	B99	DR4
IL4			

List of commonly expressed genes in the infected samples (E1b/wtp53 and E1b/mtp53) after normalization with H1299 cellular RNA.

SEMA3F	CDK2	PCNA	SOX2	P53i3	STAT1
BAI1	CDK4	p53BP1	LIN28B	IGFBP6	TADA3
KAI	CDK6	XPC	CD44	ATF3	TSC1
TP73	CDK9	AXL	NOTCH	DNMT1	XRCC5
CDC6	CDK8	Bcl-6	KLF4	EGR1	B2M
BTG2	CCNB2	p53AIP1	SIRT1	FADD	HPRT1
CHEK1	cyclin A1	BCL-2	LDHA	FOXO3	IL2
CHEK2	CCNE2	CASP3	ASCL4	HDAC1	IFNB1
TSC2	CyclinD3	CASP6	VEGFA	HK2	PCBP4
BRCA2	CDC7	CASP9	IGF1R	JUN	MSH2
BRCA1	cyclin E1	SIAH2	INSR	MCL1	NANOG
CDC25A	CCNG2	IGFBP3	TP53	MLH1	TNFRSF10B
CDC25C	CyclinD1	GML	Pin1	MSH2	SESN2
CDKN1A	CyclinB1	BID	mmp2	NFKB1	CDK1
E2F2	cyclin B3	CASP2	GDF15	KAT2B	GADD45G
E2F1	DDB2	WIP-1/P21	GTSE1	PRC1	ZEB1
E2F4	GADD45A	PTEN	RRM2B	PRKCA	TP53INP1
E2F3	GADD45B	EGFR	PMAIP1	PTTG1	RPRM

List of minimal change (NC) expressed genes in the infected samples (E1b/wtp53 and E1b/mtp53) after normalization with H1299 cellular RNA.

GD-AIF	B99	siah-3	P2XM	IRS2	TGF-Beta Superfamily protein
PAI	CyclinD2	CSR	MCG10	PTG2	basic transcription factor 2 p44
Maspin	Tip-1	PIG3	TauT	Wip1	Multiple exostoses type II protein
TSP1	P48	PIR121	SCOTIN	IL4	Vascular smooth muscle alpha-actin
TP63	CHK1	PUMA	p85	IL6	Collagen type XVIII alpha 1
P21 WAF1	P53R2	RPS27L	DR4	cMet	Neurofilament subunit NF-L
Snoothelin	Bak	PAG608	PTPA	BBC3	NB Thymosin beta
GOS2	SIAH-1	EF-1a	p14ARF	TNFRSF6	LIM domain protein
14-3-3s	P53DINP1	Zac-1	IGF1	SFN	cAMP activated protein kinase B
cdc2	KILLER/DR5	E124/PIG8	PA26	LISCH7	Lysosomal Mannosidase alpha B
MIHB	MIHC	Fra-2	ATR	RELA	purine rich element binding protein A
SIAH1	TNFRSF10D	Adipophilin	ABC3	VLDL	Histone 2A-like protein
CART	c-HA-ras 1	DM Kinase	DRAL-FHL2	ZNF127-Xp	Quiescin/QSCN6
NF-116	RB1				